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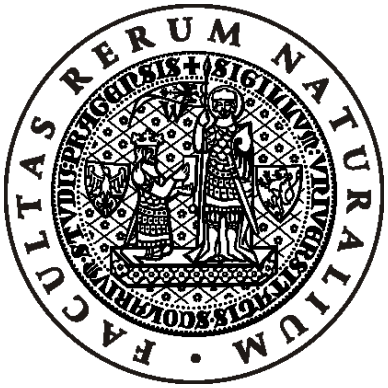

Mgr. Lukáš Děd

Vliv estrogenů a endokrinních disruptorů na savčí spermie *in vitro* a samčí reprodukční parametry *in vivo*

Effect of estrogens and endocrine disruptors on mammalian sperm *in vitro* and male reproductive parameters *in vivo*

Disertační práce

Školitelka:

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Praha, 2014

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Abstract

The endocrine system is one of the most important regulatory systems in human and animal bodies and almost all physiological processes are regulated by it. Human and animal reproduction are largely regulated by different hormones and their proper and well regulated functions are essential for successful production of the gametes, fertilization and related processes, development of the embryo and fetus, initiation of puberty and production of the offspring.

Estrogens have generally been considered as primarily female hormones. However, there is increasing evidence of their important impact on male reproductive functions. This was well documented by production of estrogen receptor knock-out male mice, which are infertile. Estrogens and estrogen receptors are also very frequent targets of endocrine disruption. Endocrine disruptors are natural or artificial compounds that, at certain doses, can interfere with the endocrine functions in human and wildlife. This interference can include specific binding to the hormonal receptor, influencing the production, elimination and transport of the natural hormones and also not well documented mechanisms including epigenetic regulations.

In the presented work, our aim was to evaluate the effects of natural estrogens and endocrine disruptors on male reproductive functions including sperm and testicular physiology, endocrine functions and epigenetics of spermatogenesis, and primordial germ cell formation.

Using a large number of techniques and different experimental approaches we uncovered an interesting effect of estrogens and endocrine disruptors with estrogenic activity on sperm physiology, especially on the dynamics of sperm capacitation. Capacitation is a physiological process that is essential for acquisition of the fertilization ability, and non-capacitated sperm are unable to fertilize the egg. According to our results, estrogens can significantly alter the dynamics of individual molecular processes playing a role during capacitation and thus potentially decrease the reproductive fitness of the exposed sperm population.

In another part of the presented work, we evaluated the effect of different endocrine disruptors on the expression of testicular genes essential for male reproductive functions. We uncovered specific changes in gene expression patterns that can further elucidate the specific effect of individual endocrine disruptors on male reproductive functions.

Finally, in the third part of the presented work we focused on the effect of endocrine disruptors on epigenetic processes playing a role during spermatogenesis and primordial germ cell formation. We uncovered a significant effect of tetrabromobisphenol A on the process of protamination and a specific effect of vinclozolin on the microRNA profiles in primordial germ cells, which were transmitted trans-generationally. These epigenetic studies open a new field of research studying how endocrine disruptors can trans-generationally influence the male reproductive fitness, and detailed analysis of these processes is essential for better understanding of the current problems in mammalian and especially human reproduction.

Souhrn

Endokrinní systém představuje jeden z nejvýznamnějších regulačních systémů v organismu člověka a živočichů, který přímo či nepřímo ovlivňuje takřka všechny fysiologické procesy. Hlavním regulačním prvkem endokrinního systému jsou hormony a jejich správná a vysoce regulovaná aktivita je základem pro úspěšnou tvorbu pohlavních buněk, oplodnění a s ním související fysiologické procesy, vývoj zárodku, zahájení puberty a tvorbu potomstva.
Estrogeny jsou v obecné rovině pokládány za především samičí hormony, zároveň však vzrůstá množství poznatků o jejich důležité roli při regulaci samčí reprodukce. Jedním z nejdůležitějších poznatků v tomto směru je zjištění, že myší samci s cíleně porušenou funkcí estrogenního receptoru alfa jsou neplodní. Estrogeny a estrogenní receptory jsou také velmi častým cílem endokrinních disruptorů. Endokrinní disruptory jsou přírodní či syntetické látky, které mohou při určitých koncentracích narušovat přirozenou hormonální regulaci člověka a živočichů. Toto narušení může zahrnovat specifickou vazbu na receptory hormonů, vliv na jejich produkci, eliminaci a transport a také nepříliš dobře zdokumentované mechanismy ovlivňující epigenetické regulace.
Naším cílem v této práci bylo zhodnotit vliv přirozených estrogenů a endokrinních disruptorů na samčí reprodukci, a to především na fysiologické procesy ve spermii a testes a epigenetické procesy odehrávající se během procesu spermatogenese a tvorby primordiálních zárodečných buněk.
Za použití široké škály experimentálních metod jsme odhalili zajímavý vliv přirozených estrogenů a endokrinních disruptorů na fysiologické procesy probíhající ve spermiích, a to především na proces jejich kapacitace. Kapacitace je fysiologický proces, během něhož ejakulovaná spermie získává schopnost oplodnit vajíčko. Bez správného průběhu kapacitace tedy spermie pozbývá schopnost oplození. Dle našich výsledků estrogeny v průběhu kapacitace významně ovlivňují dynamiku jednotlivých molekulárních dějů a ve výsledku mohou snížit oplozovací potenciál příslušné populace spermií.
V další části předkládané práce jsme hodnotili vliv různých endokrinních disruptorů na expresi testikulárních genů nezbytných pro regulaci samčí reprodukce. Zde jsme odhalili specifický vliv jednotlivých látek na expresní profily v testikulárních buňkách a tyto specifické vlivy mohou pomoci při objasňování mechanismů účinku jednotlivých látek na samčí reprodukci.
Konečně v naší poslední studii jsme se zabývali vlivem endokrinních disruptorů na epigenetické procesy hrající významnou roli ve spermatogenesi a tvorbě primordiálních zárodečných buněk. Zde jsme odhalili významný vliv tetrabrombisfenolu A na proces výměny histonů za protaminy a specifický vliv vinklozolinu na mikroRNA profil primordiálních zárodečných buněk, přičemž tento profil byl pozměněn napříč třemi generacemi. Tyto nové epigenetické studie otevírají nové pole bádání a mohou vyústit v lepší pochopení toho, jakými mechanismy endokrinní disruptory transgeneračně ovlivňují rozmnožovací schopnost samců. Hluboká analýza těchto mechanismů je pak jedním ze základních předpokladů našeho porozumění současným problémům v lidské reprodukci.

Abbreviations

ACR	acrosin gene	protein coding gene
ACR.2	anti-acrosine antibody	monoclonal antibody
Actb	β-actin (gene)	cytoskeleton
Anapc11	anaphase-promoting complex subunit 11	expressed during Meiosis I
AR	acrosome reaction	in sperm cells
BALB/c	laboratory-bred strain of the house mouse	albinotic
Blimp1	PR domain zinc finger protein 1	repressor of beta-interferon
bp	base pairs (in PCR metric)	nucleotide pairs
BSMAP	whole genome bisulfite sequence mapping program	
bw	body weight	
Ccna1	cyclin-A1 (spermatocyte specific)	expressed in spermatocytes
cDNA	complementary DNA	
c-kit	mast/stem cell growth factor receptor	cytokine receptor
CpG	C-phosphate-G DNA site	DNA region
Cq	quantification cycle	qPCR metric
Cs	cytosines (in DNA methylation analysis)	nucleotides
CTC	chlortetracycline	fluorescent compound
Cyp19A1	gene for aromatase (enzyme)	estrogens synthesis
DAPI	4',6-diamidino-2-phenylindole	fluorescent stain (DNA)
DNA	deoxyribonucleic acid	nucleic acid
Dnmt1	DNA (cytosine-5)-methyltransferase	DNA methyltransferase
dpc	day(s) post coitum	
dUTP	deoxyuridine 5'-triphosphate	nucleotide
E1	estron	natural estrogen
E3	estriol	natural estrogen
E2	17B-estradiol	natural estrogen
ED(s)	endocrine disruptor(s)	
EE2	17A-ethinylestradiol	synthetic estrogen
Eps8	epidermal growth factor receptor kinase substrate 8	
ER(s)	estrogen receptor(s)	
ERα (ERa)	estrogen receptor alpha (1)	protein
ERβ (ERb)	estrogen receptor beta (2)	protein
ESR1	estrogen receptor 1 (alpha)	gene
ESR2	estrogen receptor 2 (beta)	gene
F0	zero generation	
F1	first generation	
F2	second generation	
F3	third generation	
FITC	fluorescein isothiocyanate	fluorescent compound
FSH	follicle-stimulating hormone	hormone (pituitary)
GPR30	G protein-coupled receptor 30	membrane ER
Grth	gonadotropin-regulated testicular helicase	RNA helicase

HCl	hydrochloric acid	strong mineral acid
Hs-14	anti-acrosomal antibody	monoclonal antibody
Icap1	integrin beta-1-binding protein 1	Sertoli cell marker
IHC	immunohistochemistry	method
Kdm4a	lysine-specific demethylase 4A	histone demethylase
let-7	lethal-7	microRNA molecule
Lin28	lin-28 homolog A	microRNA-binding protein
M2	culture medium	mouse embryo tested
Mas1	Mas proto-oncogene	
MEHP	mono(2-ethylhexyl)phthalate	phthalate, ED
miR(*number*)	specific microRNAs	
miRNA(s)	microRNA(s)	non-coding RNA(s)
mmu-miR(s)	microRNA(s) (mus musculus originated)	
NIS	Nicon instruments	company name
P	protamines	sperm nuclear proteins
P1	protamine 1	sperm nuclear protein
P2	protamine 2	sperm nuclear protein
PBS	phosphate buffered saline	buffer solution
PCR	polymerase chain reaction	DNA amplification method
PGC(s)	primordial germ stem cell(s)	
Prm1	protamine 1	sperm nuclear protein
Prm2	protamine 2	sperm nuclear protein
pY	phosphotyrosine	modified amino acid
qPCR	quantitative polymerase chain reaction	DNA amplification method
RNA	ribonucleic acid	nucleic acid
RRBS	reduced representation bisulfite sequencing	analytical method
Sik1	serine/threonine-protein kinase	protein kinase
Sox9	transcription factor Sox-9	Sertoli cell marker
Spata2	spermatogenesis-associated protein 2	FSH-dependent
SSEA1	stage-specific embryonic antigen-1	stem cell marker
Sycp1	synaptonemal complex protein 1	expressed in spermatocytes
Sycp3	synaptonemal complex protein 3	expressed in spermatocytes
TBBPA	tetrabromobispheol A	flame retardant
TNP1	spermatid nuclear transition protein 1	expressed in spermatids
TNP2	spermatid nuclear transition protein 2	expressed in spermatids
Tris	tris(hydroxymethyl)aminomethane	organic compound
Ts	thymines (in DNA methylation analysis)	nucleotides
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling	
TyrP	tyrosine phosphorylation	protein modification
VCZ	vinclozolin	fungicide
Vegfa	vascular endothelial growth factor A	growth factor
WHO	World Health Organization	agency (United Nations)
Wt1	Wilms tumor protein	Sertoli cell marker
ZEA	zearalenone	product of some fungi

1. Introduction:

1.1. Endocrine system, estrogens and estrogen receptors

The endocrine system is one of the most important regulatory systems in human and animal bodies and almost all physiological processes are regulated by it. The endocrine regulation is also tightly connected to the neural and immune systems (Haddad et al., 2002). The most primitive endocrine systems consist of neurosecretory cells, which release the hormones directly into the blood or other body fluids (Tessmar-Raible et al., 2007). In higher animals, and especially vertebrates, the endocrine system consists of multiple specialized organs and appropriate cell types. These specialized organs and cell types produce tens or hundreds of specialized hormones, which finely regulate appropriate physiological processes.

Reproduction of mammals is a highly complex process in which almost all parts of the endocrine system play some role (Atkinson et al., 2007; Bhasin et al, 2002). Furthermore, the specialized endocrine organs and hormones evolved primarily as regulators of the reproductive process. Despite their complex effect on mammalian physiology, their primary function is to regulate the processes such as production of gametes (spermatogenesis and oogenesis), sex determination, fertilization, implantation, birth, initialization of puberty, etc.

During evolution, the specialized endocrine organs and hormones evolved to be produced primarily in one sex only. In vertebrates, sex or gonadal steroids are represented by androgens in males and estrogens in females.

Despite the fact that estrogens are primarily responsible for the regulation of reproductive physiology in females, increasing evidence suggests their essential role in male reproductive functions (Carreau et al., 2007, 2011). This was well documented by production of estrogen receptor knock-out male mice, which are infertile (Eddy et al., 1996). Natural estrogens are steroid hormones and in mammals there are three major types of them – estrone, estriol, and the most potent one – 17B-estradiol (Figure 1).

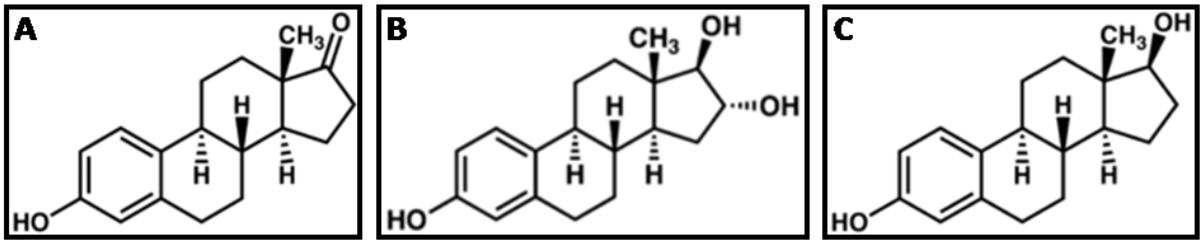

Figure 1. Structures of estrone (A), estriol (B) and 17B-estradiol (C)

Beside natural estrogens, some synthetic analogs were developed by pharmaceutical industry such as 17A-ethinylestradiol (Djerassi, 2006). These compounds have higher stability and are able to overcome the first pass through the liver tissue, where most of the natural estrogens are eliminated (Bolt, 1979).

Estrogens act at the molecular level by two major ways – genomic and non-genomic. In the classical genomic pathway, estrogens bind to their nuclear receptors in the cytoplasm (Dahlman-Wright et al., 2006). After binding, the estrogen receptors translocate into the nucleus and bind to DNA to regulate gene expression. In most mammals, there are two major types of estrogen nuclear receptors – ESR1 and ESR2. Both ESR1 and ESR2 are widely expressed in various tissues; however there are some notable differences in their expression patterns. In the male reproductive tract, the expression of individual ER variants is dependent on the appropriate cell and tissue type and on the developmental stage (Figure 2).

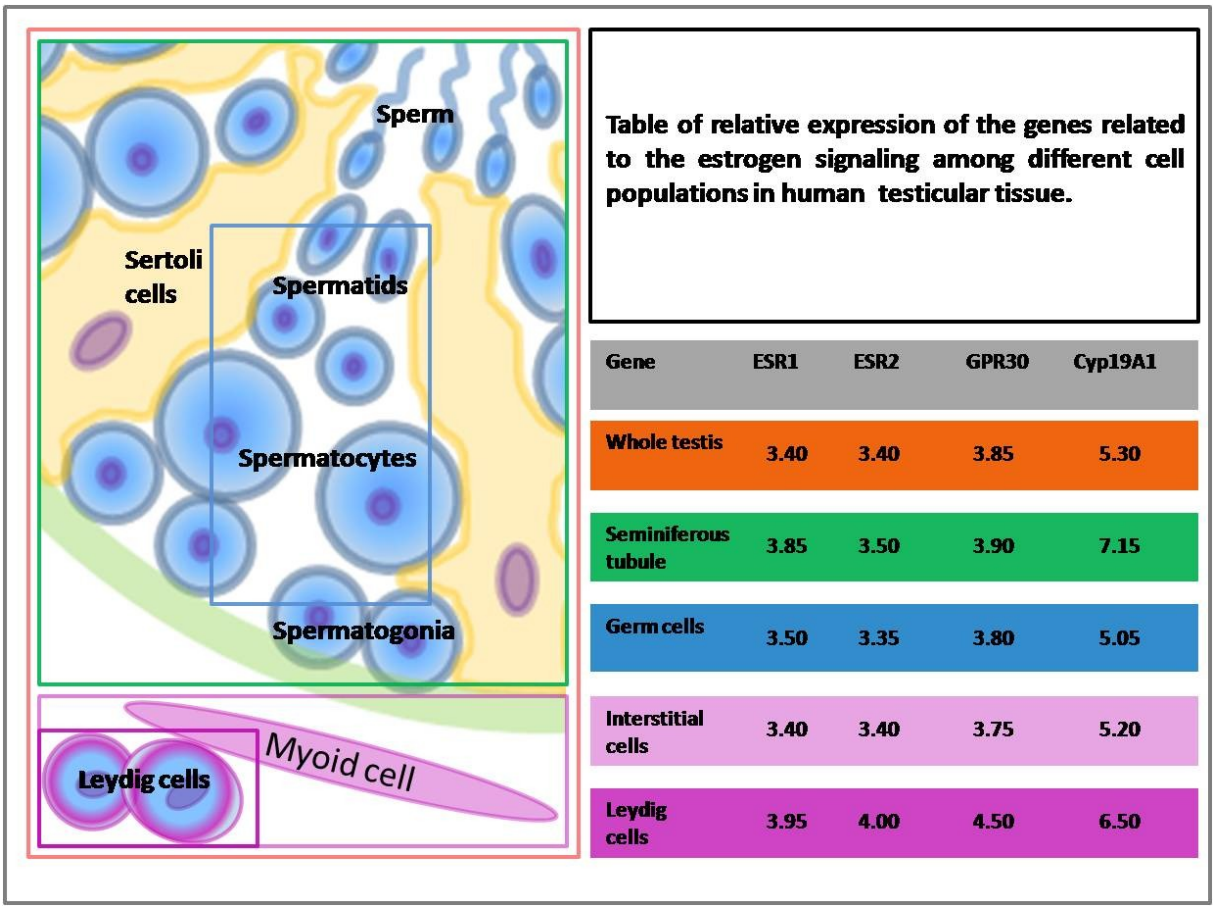

Table of relative expression of the genes related to the estrogen signaling among different cell populations in human testicular tissue.

Gene	ESR1	ESR2	GPR30	Cyp19A1
Whole testis	3.40	3.40	3.85	5.30
Seminiferous tubule	3.85	3.50	3.90	7.15
Germ cells	3.50	3.35	3.80	5.05
Interstitial cells	3.40	3.40	3.75	5.20
Leydig cells	3.95	4.00	4.50	6.50

Figure 2. Overview of the estrogen signaling-related gene expression in testicular tissue (expression data obtained from BioGPS database).

Beside the classical genomic pathway, estrogens can also act via relatively newly discovered non-genomic pathways. Signaling via a non-genomic pathway can be mediated both by

"classical" estrogen receptors ESR1 and ESR2 associated with the membranes (Zivadinovic et al., 2005) and by specialized G-protein-coupled receptors (O'Dowd et al., 1998). In the membrane, ERs can interact with caveolin and form complexes with striatin and receptor tyrosine kinases. This interaction may lead to increased levels of Ca^{2+} and protein phosphorylation. Furthermore, estrogens can act via G-protein-coupled receptor GPR30 (Prossnitz et al., 2007). The molecular function and localization of this receptor is still object of controversy. Despite the fact that a large number of reports describe GPR30 as a membrane estrogen receptor localized in endoplasmic reticulum, there are also some contrary studies indicating that GPR30 does not act as the estrogen receptor (Langer et al., 2010). In 2014, the presence of the GPR30 receptor was confirmed in the human and pig sperm (Rago et al., 2014).

1.2. Endocrine disruptors

Endocrine disruptors are natural or artificial compounds that, at certain doses, can interfere with the endocrine functions in human and wildlife (Crisp et al., 1998). This interference can include specific binding to the hormonal receptor, influencing the production, elimination of and transport of the natural hormones and also not well documented mechanisms including epigenetic regulations.

Estrogens and estrogen receptors are very frequent targets of endocrine disruption (Safe et al., 2010). All steroidal hormones are produced from the common precursor – cholesterol – and their specific biological activity depends on different modifications of the cyclopentanoperhydrophenanthrene scaffold. Because of a large variety of modifications of the sterane scaffold, different steroid compounds and steroid receptors are able to cross-react non-specifically. Furthermore, the steroid receptor protein family probably evolved from the common ancestor which was highly specific for estrogen (Eick and Thornton, 2011).

Under physiological conditions, the major portion of natural compounds with estrogenic activity (estrogenic xenobiotics, xenoestrogens) is eliminated during the first pass effect in the liver tissue by the P450 system (Jakoby and Ziegler, 1990). The liver detoxification system is able to highly increase its functional potential by activation of non-specific steroid receptors, which then bind to the promoter areas of the enzymes participating in the P450 system and activate their expression (Mikamo et al., 2003). However, the estrogen contraceptives, such as 17A-ethinylestradiol, are more resistant to the degradation by P450 in comparison to their natural counterparts like 17B-estradiol (Guengerich, 1990). Furthermore, some endocrine

disruptors can enter the body and blood circulation by the respiratory system or skin and thus bypass rapid elimination in the liver portal system.

In presented study, the effects of four major endocrine disruptors were evaluated (Figure 3). 17A-ethinylestradiol is an orally bioactive estrogen, derivative of 17B-estradiol, used in many formulations of combined oral contraceptive pills (Djerassi, 2006). It is famous as an important environmental pollutant with a significant effect especially on fish and water life (Lange et al., 2009). TBBPA is used as a reactive and additive flame retardant especially in modern electronic devices (WHO, 1995). The major way of exposure is inhalation of the house dust. Zearalenone is an organic compound produced by some Fusarium and Gibberella species (Zinedine et al., 2007). It acts as a strong estrogenic metabolite and may cause infertility in wildlife and livestock, especially in swine (Benzoni et al., 2008).

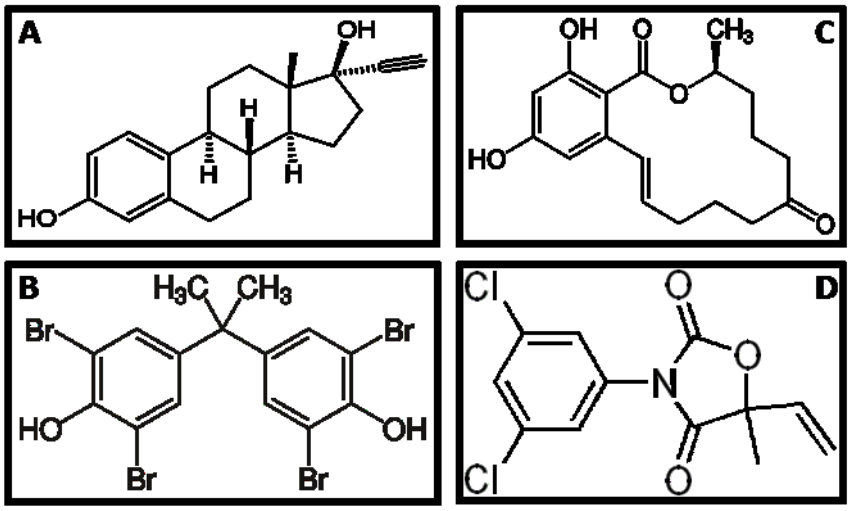

Figure 3. Structures of 17A-ethinylestradiol (A), TBBPA (B), Zearalenone (C) and Vinclozolin (D).

Vinclozolin (VCZ) is a common dicarboximide fungicide. It is known to act as ED with anti-androgenic activity (Kelce et al., 1997).

1.3. Sperm physiology and capacitation

Since their release into the lumen of the seminiferous tubule, highly differentiated sperm cells undergo a series of physiological and molecular changes that give them the ability to fertilize the egg. These changes especially involve sperm maturation in the epididymis and the process of sperm “capacitation”. Capacitation involves the physiological changes that the sperm must undergo in the female reproductive tract or *in vitro* to obtain the ability to bind,

penetrate and fertilize the egg (Austin 1951; Austin and Bishop 1958; Chang 1951). As a physiological process, capacitation is based on many molecular mechanisms (Figure 4). These mechanisms include changes in the intracellular calcium concentration (Reyes et al., 1978), rearrangement of the acrosomal matrix (Peknicova et al., 1994), rearrangement of the sperm cytoskeleton (Breitbart et al., 2005), phosphorylation of sperm proteins (Seshagiri et al., 2007) and changes in the sperm plasmatic membrane (Cross, 1998). From the time of discovery of the capacitation process, several methods have been developed to characterize this complex biological process. All these methods detect changes in the molecular processes enumerated previously.

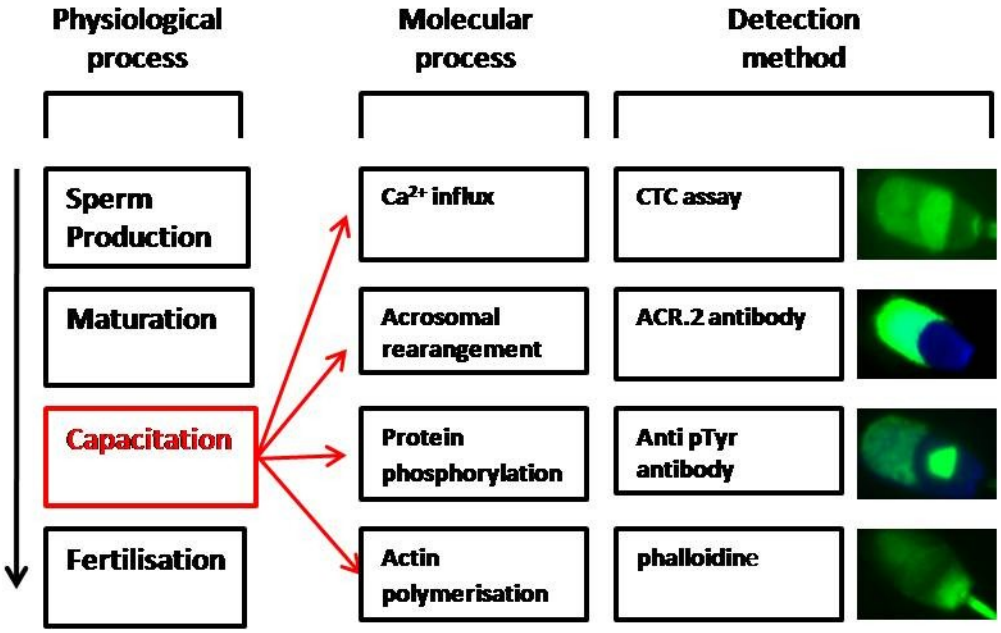

Figure 4. Four major physiological processes taking place in the testes, epididymis and female reproductive tract during sperm life with the description of the molecular processes playing a role during capacitation and their detection techniques

The CTC method developed by Ward and Storey is able to detect the redistribution of intracellular calcium in the sperm head during the capacitation process (Ward and Storey, 1984). Anti-acrosine antibody (ACR.2) is able to recognize the rearrangement of the acrosomal matrix by detecting changes in the accessibility of the acrosin epitopes (Peknicova et al., 1994). The higher accessibility of the acrosin epitopes is a significant marker of the capacitation progress. Phalloidine (fluorescein isothiocyanate-conjugated phalloidine, FITC-phall) binds to f-actin, whose amount significantly increases during the capacitation progress (Brener et al., 2003). Finally, the capacitation process involves phosphorylation of various proteins (Kalab et al., 1998), which is detectable by fluorescein isothiocyanate-conjugated

anti-phosphotyrosine (pY) antibody (anti-pY), and rearrangement of the plasmatic membrane detectable e.g. by anti-caveolin antibody (Travis et al., 2001).
Since capacitation is a process that is specific for mammalian species, it represents a specific target for EDs in this taxonomic unit.

1.4. Spermatogenesis and gene expression

Spermatogenesis is a process during which sperm are produced from spermatogonia, primary and secondary spermatocytes, and spermatids (Sharpe, 1994). Spermatogenesis as the cellular process includes both mitotic and meiotic processes. In mammals, spermatogenesis takes place in specialized organs – male gonads – testes (Figure 5). The whole process starts with specialized stem cells – spermatogonia. Spermatogonia undergo mitotic division in the process of their self-renewal and production of primary spermatocytes. Primary spermatocytes undergo the process of heterotypic division (Meiosis I) and give rise to secondary spermatocytes. These cells undergo the process of homotypic cell division (Meiosis II) and thus round spermatids are created.

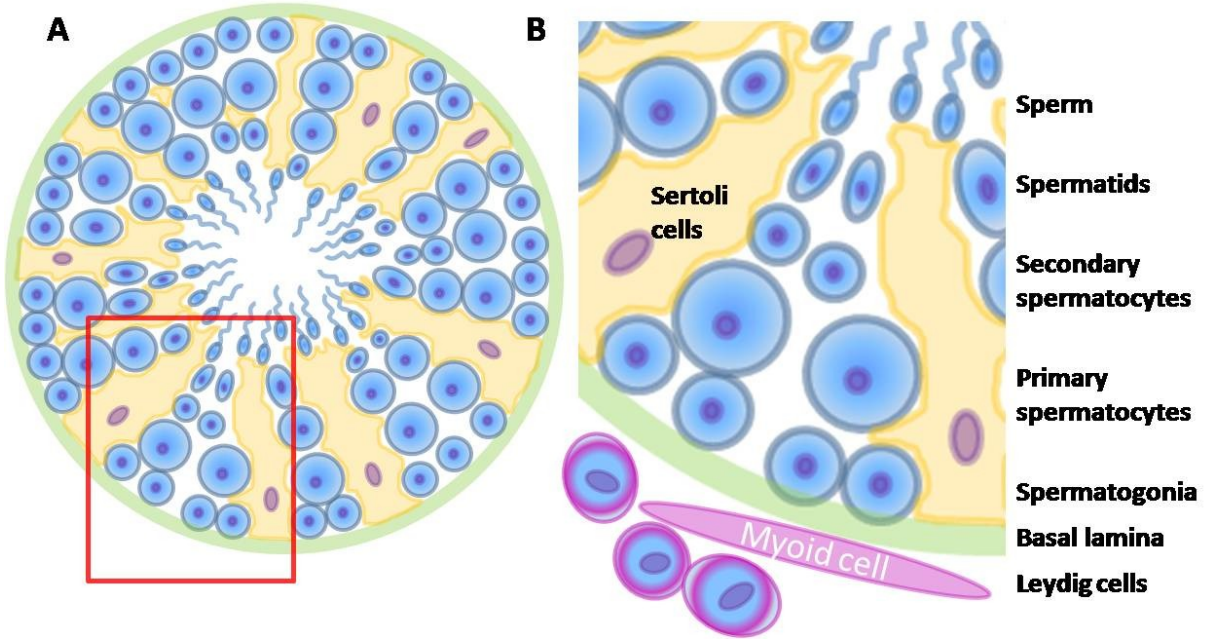

Figure 5. Scheme of the seminiferous tubule (A) and its higher magnification with the description of individual cell types inside and outside the seminiferous tubule (B)

The process in which round spermatids are transformed into sperm is called spermiogenesis. During this process, spermatids lose a significant part of their volume with appropriate

organelles and cytoplasm, histones in the nucleus are replaced by protamines and the sperm tail is developed. Most of the removed parts are processed by specialized Sertoli cells. These cells, which are present inside the seminiferous tubules, also significantly support the whole process of spermatogenesis (Griswold and McLean, 2006).

Spermatogenesis is a very specialized process in which many unique genes play a role. These genes are often connected with the process of meiosis, spermatogonial stem cell self-renewal, process of spermiogenesis and production of the compacted sperm nucleus. Therefore, some of the genes that play a role in the testicular physiology are expressed in only one type of testicular cells and can be used as a marker of these cell populations in qPCR or immunohistochemical experiments (Figure 6).

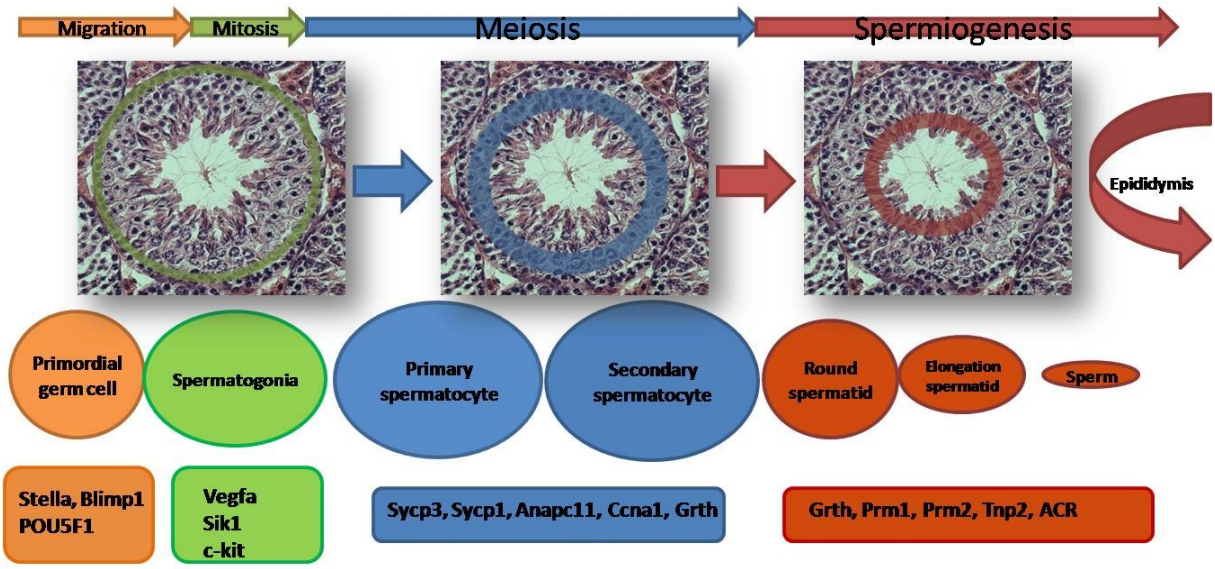

Figure 6. Scheme of individual stages of the spermatogenesis process with the appropriate cell types and selected marker genes

In spermatogonia, Vegfa, Sik1 and c-kit genes represent such gene markers. Vegfa is a gene that plays an important role in the regulation of the cell cycle (Caires et al., 2012). It is a member of the platelet-derived growth factor (PDGF) / vascular endothelial growth factor (VEGF) family and acts as glycosylated mitogen. C-kit is a cytokine receptor expressed on the surface of hematopoietic stem cells as well as other cell types. In the testis, c-kit is specifically expressed in spermatogonia (Zhang et al., 2013). It acts as a receptor tyrosine kinase type III, binds to stem cell factor named c-kit ligand and regulates the proliferation and

differentiation of spermatogonia. Spermatocytes together with oocytes are the only cells in the mammalian bodies that undergo the process of meiosis, and therefore many of their marker genes play a role in this unique process. Sycp1 (Tureci et al., 1998) and Sycp3 (Yuan et al., 2000) are structural components of the synaptonemal complex, which enables the proper chromosomal pairing and recombination during heterotypic cell division (Fraune et al., 2012). Their expression is therefore restricted to prophase of Meiosis I. Anapc11 and ccna1 genes are involved in the cell cycle regulation in spermatocytes and are expressed only in this cell type in the testis (Yang et al., 1997). On the other hand, expression of the Grth gene (Dufau et al., 2007) starts in meiotic spermatocytes, but its proper expression is also important in the later stages of the spermatogenesis process – in spermatids. Beside other functions, it is essential for the regulation of the apoptotic process, and disruption of its function leads to higher apoptotic rate in appropriate cell types. The spermatids are the last cells with active gene expression and they finally differentiate into the transcriptionally inactive sperm cells (Sharpe et al., 1994). Therefore, many genes encoding proteins that are unique for the highly specialized sperm cells are also uniquely expressed in spermatids. Those are for example genes encoding proteins participating in the process of nuclear compaction. During this process, histones are replaced by transition proteins TNP1 and TNP2 (Meistrich et al., 2003), which are subsequently replaced by protamines Prm1 and Prm2 (Balhorn, 2007). Also some other proteins that are present in specialized sperm compartments such as acrosome are expressed in the spermatid stage. It is for example acrosin, the major acrosomal protease, encoded by the ACR gene.

It is important to note that the well-orchestrated activity of individual genes is an absolute prerequisite for successful spermatogenesis, and dysfunction of some essential genes leads to disruption of the spermatogenetic process and subsequent infertility.

1.5. Primordial germ cells (PGCs) and epigenetics

Primordial germ cells (PGCs) are embryonic precursors of the germ cell lineage, which are restricted to form only sperm and oocytes following their specification from pluripotent cells (Saitou et al., 2012). PGC precursors are specified in the epiblast around 6.25 days post coitum (dpc), and around 7.25 dpc become identifiable in a 40 cell-cluster (Figure 7). Thereafter, PGCs migrate through hindgut endoderm and colonize the genital ridges at day 10.5. PGC specification depends on interactions among various molecular factors. Many of these factors are involved in the process of epigenetic regulation (Leitch et al., 2013).

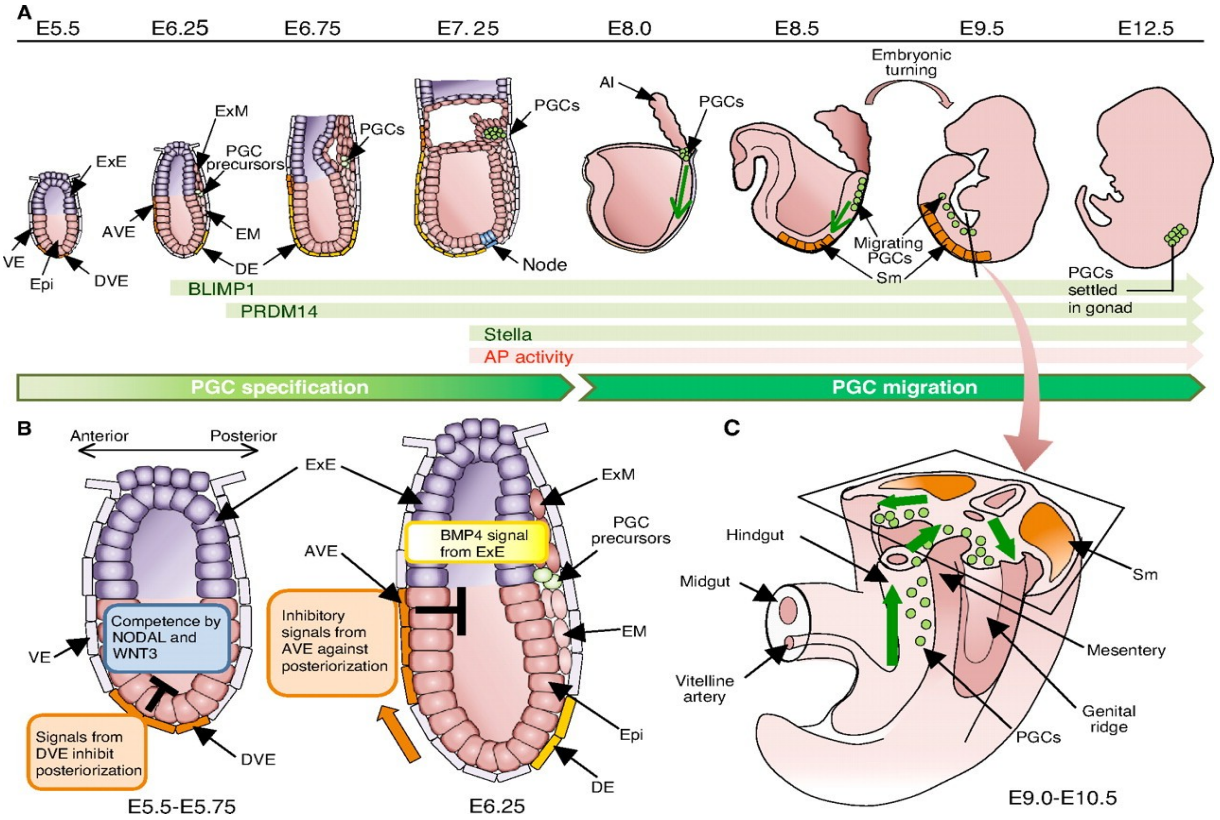

Figure 7. Description of the specification and migration of PGCs during embryonic development (Saitou et al., 2012)

In general, epigenetics is the study of heritable changes in the gene activity that are not caused by changes in the DNA sequence. More specifically, molecular epigenetics focus especially on the study of histone modifications, DNA methylation and RNA interference (Figure 8). In PGCs, some of those non-coding RNAs, especially microRNAs and their regulating proteins, play an important role during migration, differentiation and self-renewal of this specific cell type. For example, PGC specification depends on interactions among microRNA-binding protein LIN28, microRNA let-7 and BLIMP1 protein (Leitch et al., 2013). In the absence of LIN28, let-7 will bind to the 3′UTR of the Blimp1 mRNA to block its translation and prevent PGCs from developing. During PGC formation, LIN28 binds to the let-7 family pri-miRNA loop to prevent processing of these precursor forms into the mature let-7 miRNA, allowing BLIMP1 translation, and permitting PGC specification.

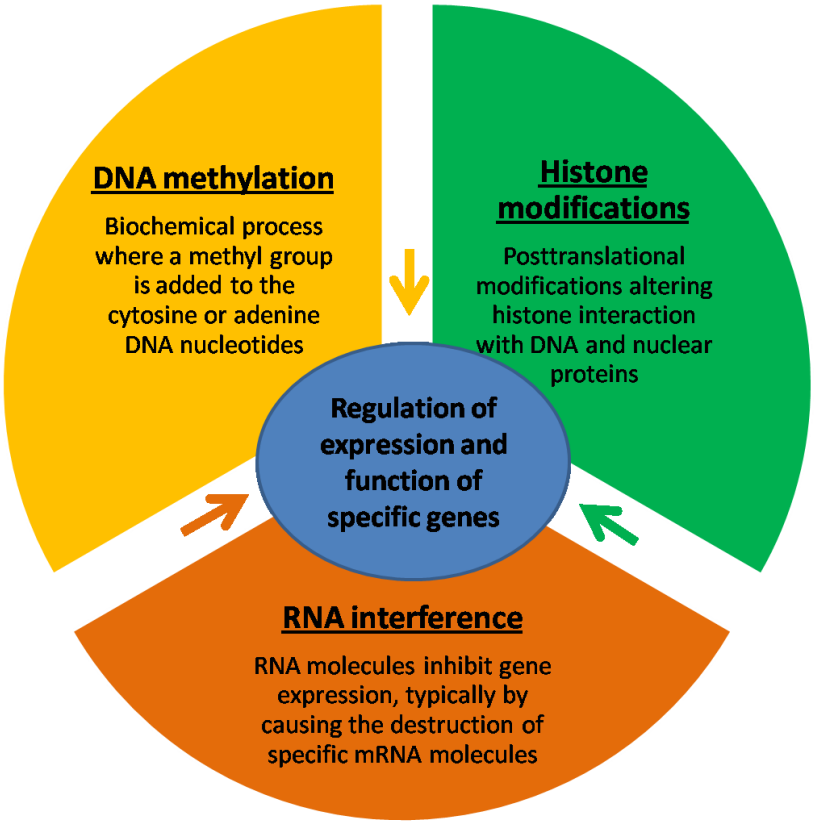

Figure 8. Three major molecular processes involved in the process of epigenetic regulation

Like other complex biological processes, PGC specification can also be deregulated by various environmental factors including EDs.

2. Aim of the work:

To evaluate the effects of natural estrogens and endocrine disruptors on male reproductive functions including sperm and testicular physiology, hormonal levels and activities, gene expression during spermatogenesis and epigenetics of primordial germ cell formation.

3. Methods:

Experimental models: mice and boar

3.1. Sperm capacitation (mice)

Sperm from the distal regions of cauda epididymis were released into M2 fertilizing medium under paraffin oil at 37°C in 5% CO_2. The released sperm were assessed for motility and viability. The sperm stock was diluted to the required concentration ($5x10^6$/ml) into M2 medium under paraffin oil and incubated at 37°C. To detect the influence of estrogens on sperm capacitation, sperm were added to the medium with different concentrations of selected estrogens (0.02, 0.2, 2, 20, and 200 ng/ml). Sperm capacitated in M2 medium without added estrogens were used as a control. Sperm samples were collected at different experimental times of *in vitro* capacitation. Collected sperm were washed in PBS and a smeared section was prepared.

3.2. Chlortetracycline fluorescent assay (CTC assay)

Sperm were re-suspended in PBS and mixed with an equal volume (45 μl/45 μl) of chlortetracycline CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris-HCl, pH 7.8) and incubated for 30 min. Cells were then fixed by 8 μl of 12.5% paraformaldehyde in 0.5 mol/l Tris-HCl (pH 7.4). After incubation, sperm suspension was smeared onto a glass slide and covered with a cover slip.

3.3. Indirect immunofluorescence (sperm cells)

Monoclonal antibody against intra-acrosomal protein (Hs-14) was used to assess the integrity of the acrosome. This antibody was prepared in our laboratory and is routinely used to test the acrosome state. Epididymal spermatozoa were dropped on glass slides, air dried and fixed for 10 min with acetone. After rinsing with PBS, the slides were incubated overnight at 4°C with monoclonal antibody (diluted to an immunoglobulin concentration 20 μg/ml). After thorough washing with PBS, the slides were incubated with anti-mouse IgM (chain specific) fluorescein isothiocyanate (FITC) conjugate (Sigma, Prague, Czech Republic) diluted 1:128 in PBS and incubated for 60 min at 37°C, washed with PBS and distilled water, and mounted in Vectashield H-1200 DAPI (Vector Laboratories, Burlingame, CA) for DNA visualization.

3.4. Quantitative polymerase chain reaction (qPCR, BIOMARK)

Gene expression analysis was performed in BioMark (Fluidigm, San Francisco, CA), which enables performing a large number of real-time PCR reactions in a single run. Before performing BioMark analysis the samples were pre-amplified. The pre-amplification reaction was done as follows: 2 µl of cDNA (10 ng RNA/µl) was mixed with 1.2 µl of 208 nM primer mix (all primers were mixed together, final concentration of each primer 25 nM), 5 µl of iQSupermix (BioRad, Prague, Czech Republic) and 1.8 µl of H_2O. The mixture was first incubated for 10 min at 95°C, then followed 18 cycles of 15 s at 95°C, and finally 4 min at 59°C. Pre-amplified cDNA was diluted 20×. The real-time PCR reactions were carried out in GE Dynamic array 48x48 in a BioMark HD System (Fluidigm, SanFrancisco, California). Five µl of Fluidigm sample premix consisted of 1 µl of 20× diluted pre-amplified cDNA, 0.25 µl of 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), 2.5 µl of SsoFastEvaGreen Supermix (Bio-Rad, Czech Republic), 0.1 µl of 4× diluted ROX (Invitrogen, USA) and 1.15 µl of RNase/DNase-free water. Each assay premix consisted of 2.5 µl of 10 µM primers (forward and reversed at a final concentration of 500 nM) and 2.5 µl of DAAssay Loading Reagent (Fluidigm, USA). Thermal conditions for qPCR were: 98°C for 40 s, 40 cycles of 95°C for 10 s, and 60°C for 40 s. The β-actin (Actb) reference gene was selected from several reference gene candidates by Normfinder (GenEx Enterprise, Mul-tiD Analyses, Sweden). The data were collected using BioMark 3.1.2 Data Collection software and analyzed by BioMark Real-Time PCR Analysis Software 3.1.3. (Fludigm, USA). The cut-off value for Cq was set at 25 and values higher than that were replaced by the Cq value of 25. The missing data were filled with maximum of a column plus 1. Data were normalized with actin. The fold change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method for each sample and then expressed as the mean of all these fold changes. The control was set at 100% and experimental samples were compared to the control.

3.5. Extraction of sperm nuclear proteins

For protamine extraction 5 x 10^6 of mouse epididymal sperm cells were used. The spermatozoa were washed in PBS and the pellet was resuspended in 100 µl of 0.5% Triton X-100, 20 mM Tris, 2 mM $MgCl_2$ and centrifuged at 8940 g, 5 min, 4°C. After centrifugation the samples were treated as previously described (de Yebra & Oliva 1993), except that instead of iodoacetate treatment we performed treatment with 0.8% vinylpyridine (commercial information) for 30 min at 37°C to further inhibit formation of cysteine disulfide bonds. Finally, each sample was re-suspended in 10 µl of sample buffer containing 5.5 M urea, 20%

β-mercaptoethanol and 5% acetic acid. The DNA remaining in the pellet after the extraction of the nuclear proteins with 0.5 M HCl was extracted and quantified after 0.5 M perchloric acid hydrolysis (90°C, 20 min) and absorbance determination at 260 nm, measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, Delaware).

3.6. Separation and analysis of sperm nuclear proteins

Basic nuclear proteins were analyzed using acid-urea polyacrylamide gel containing 2.5 M urea, 12.5 mM thiourea, 0.9 M acetic acid, 15% acrylamide, 0.1% bis-acrylamide and 0.12% H_2O_2. After polymerization, 2 µl of each sample was loaded and the gel was electrophoresed in 0.9 M acetic acid buffer for 90 minutes at 110 V. Different known quantities of a human protamine standard from a pool of human normozoospermic sperm samples (0.435, 0.87, 1.74 and 2.61 µg) were added in each acid urea electrophoretic gel. The gels were stained with EzBlue staining reagent (Sigma, Prague, Czech Republic) following the manufacturer's instructions. The stained gels were scanned and the intensity of the bands corresponding to protamine 1 and protamine 2 was quantified with QuantityOne software (Bio-Rad Laboratories, Hercules, California). The data obtained from QuantityOne software were used for calculating the P1/P2 ratio. A regression curve was obtained from the different concentrations of human protamine standard in order to calculate the total amount of protamines (P1 + P2) in each sample. P/DNA ratios were calculated.

3.7. TUNEL and immunocytochemical analysis

Paraffin-embedded tissue sections were rehydrated in water, re-fixed in 4% formaldehyde, incubated in proteinase K solution (20 µg/ml) for 5 min, washed two times in PBS, incubated for 10 min in an equilibration buffer and finally exposed for 1 h to the labeling buffer containing both FITC-labeled dUTP and terminal deoxynucleotidyl transferase. Control samples without the terminal deoxynucleotidyl transferase enzyme or treated by DNase were also prepared. TUNEL-labeled samples were then washed in saline–sodium citrate buffer and mounted in Vectashield with DAPI to visualize the nuclei. TUNEL-positive cells were counted in seminiferous tubules by fluorescent microscopy. Ten tissue slides were analyzed per testis and the numbers of TUNEL-positive cells were normalized per area and averaged as technical replicates. Ten different testes were analyzed in each group. In all samples, the number of TUNEL-positive cells was analyzed by NIS Elements picture analyzer. The embryonic testes were dissected from 13.5 dpc embryos and fixed in 4% formaldehyde in PBS. We prepared paraffin-embedded tissue sections (5 µm thick). PGCs were labeled by

anti-SSEA1 antibody (Abcam, ab16285). In brief, the tissue sections were deparaffinized, rehydrated and incubated in PBS for 5 min. Antigen retrieval was performed by heating in citrate buffer (IHC world general protocol) and HistoReveal (Abcam) according to the manufacturer's protocol (5 min incubation at RT) and protein blocking was performed by Protein Block (Abcam) during 10 min at RT. The slides were subsequently incubated with the primary antibody (anti-SSEA1, Abcam, ab16285, 10 µg/ml) at 37 °C for 1 h. After washing in PBS, slides were incubated with the secondary antibody (Goat Anti-Mouse IgM H&L, Abcam, 10 µg/ml) at 37 °C for 1 h. After washing in PBS and water, the tissue slides were used for TUNEL assay according to the protocol described above with some modifications (avoiding formalin fixation and proteinase K digestion). Subsequently, slides were mounted with Vectashield (Vector) (DAPI counterstaining) and analyzed by fluorescent microscopy. The numbers of TUNEL-positive and SSEA1-positive cells were counted with NIS Elements picture analyzer and normalized to the sample area.

3.8. DNA methylation

Final RRBS libraries were amplified with 17 cycles of PCR and subjected to paired-end sequencing (2 x 75 bp) in an Illumina HiSeq2000 apparatus. Sequencing reads were cleaned with Trim Galore and aligned to the mouse mm10 genome with BSMAP, allowing four mismatches. We only retained reads with a unique best hit in the genome. Methylation percent values were calculated with BSMAP as the ratio of the number of Cs over the total number of Cs and Ts. RRBS was performed on pools of PGCs purified from 30 testes. For sperm, RRBS was performed in duplicates on sperm pooled from three exposed animals, as well as sperm samples pooled from unexposed animals. To find differences in DNA methylation, we averaged methylation scores in 400 bp tiles and searched for tiles with at least 3 CpGs and 25% methylation difference. All data processing and representation were performed with the R software using custom-developed scripts (http://www.r-project.org). For gene-specific analysis of DNA methylation, we performed bisulfite conversion of genomic DNA with the Epitect kit (Qiagen). Subsequently, PCR amplifications were performed at regions of interest followed by cloning.

4. Results and discussion

4.1. Effect of natural and synthetic estrogens on the capacitation process in boar and mice sperm (Supplements 1, 2, 3).

In these studies, we analyzed the effect of various estrogens on the capacitation status of the sperm. In the first study (Supplement 1) we analyzed the effect of three natural (E1, E2, E3) and one artificial (EE2) estrogens at various concentrations on the capacitation progress of boar sperm *in vitro*. Using flow cytometry approach and our own detection technique of the anti-acrosin antibody, ACR.2, we uncovered that the most potent estrogen E2 at the 1 μM concentration has a pro-capacitation effect on the boar sperm. The highest difference in the number of capacitated and non-capacitated sperm cells was observed at 120 min of *in vitro* capacitation. At this time-point, we carried out the experiments and performed the analysis also with other estrogens at six different concentrations including the physiologically relevant hormonal levels. We also used another detection technique (CTC) to confirm our experimental results. During the analysis of the results from one animal (boar A), we observed the first statistically significant difference between the control and experimental group (E2) in the number of capacitated sperm at the 5 nM concentration. In the same animal, all tested estrogens had a significant effect at 100 nM concentration. To test the reproducibility of our results, we decided to perform the analysis on seven additional animals (boars B-H). Surprisingly, we detected a significant effect of 1 μM E2 on the capacitation progress only in one half of the animals. In the animals with the negative reaction of the sperm population on E2 at 1 μM concentration, only very high concentrations of estrogens (10 μM – 100 μM) had a significant effect.

To overcome the problems with inter-individual heterogeneity in the boars, we decided to perform a similar experiment in mice. In our second study (Supplement 2) we analyzed the effect of the same estrogens on the capacitation progress of mouse sperm using the immunoflourescent and western blot detection techniques with anti-phosphotyrosine antibody (anti-pY). The first interesting result was the detection of ERβ in mouse spermatozoa (BALB/c) because it was the first detection of this receptor in mouse sperm. This receptor was localized in the apical area as a thin crescent of the sperm head. In the capacitation part of our experiment, we obtained similar results to our boar study. The individual estrogens had a pro-capacitation effect on the mouse sperm during *in vitro* capacitation. Due to the faster progress of capacitation in mice, we observed the first significant differences in the number of

pY head-positive sperm cells after 30 min of *in vitro* capacitation. The effect of individual estrogens on the mouse sperm capacitation progress differed according to their concentration and time of incubation. The higher concentrations of estrone, E2 and EE2 increased TyrP. Estriol acted differently; the increase of TyrP was especially noticeable at low concentrations. E2 showed the longest influence on the processes in the sperm head. To test the reproducibility of our results we again used a different experimental technique – western blot detection with anti-phosphotyrosine antibody (anti-pY). The results showed a time-dependent increase of the level of protein TyrP of a molecular weight of 40-120 kDa. The maximum level of TyrP was reached, especially at higher times of capacitation, but this observation resulted from the fact that the level of TyrP is displayed not only in the sperm head, but also in the flagellum, which was not the object of our immunofluorescent analysis. In another part of our study we also studied the effect of estrogens on the ability of sperm to undergo calcium ionophore-induced AR. Estrogens significantly reduced the percentage of sperm that undergo AR in appropriate detection times. E1, E2 and E3 showed a very similar effect on the the sperm AR. They significantly reduced the percentage of sperm undergoing AR in the low capacitation times with a short induction of the AR. AR was influenced by the three highest concentrations of 17B-estradiol and estrone, and for estriol by the three lower concentrations. On the other hand, EE2 expressed a different pattern of AR influence with a protracted peak of inhibition activity.

Having the knowledge about the *in vitro* effect of estrogens on sperm capacitation, we also wanted to know what happened to the sperm population after more natural *in vivo* exposure to estrogens (Supplement 3). During this study, one group of males was exposed to 17B-estradiol at the concentration of 20 ng/ml during puberty from 4th to 7th week of age (Pubertal group). The second group of males was exposed continuously from birth to the 12th week of age (Continuous group). The control group of mice was not exposed to 17B-estradiol. During direct analysis of the epididymal sperm by CTC and anti-pY antibody, we observed a significantly higher number of capacitated sperm in both experimental groups compared to the control one. The initial differences of the sperm head TyrP were then propagated during *in vitro* capacitation. Contrary to these results, no significant differences in the capacitation status of the testicular sperm were detected by anti-pY antibody. We therefore concluded that *in vivo* exposure to E2 leads to premature sperm capacitation in the cauda epididymis.

To date, the mechanisms of the estrogen effects on sperm physiology have not yet been elucidated. To our opinion, these mechanisms can be mediated by receptors and/or effect on

membrane biophysics. In our first study, we observed different effects of estrogens on the capacitation progress. These effects depend on the individual animals. In some sperm samples, estrogens influence capacitation at physiologically relevant concentrations, but in other samples they do not. The effect of estrogens expressed concentration dependence even at the concentrations at which estrogens receptors are saturated. This may indicate that both receptor-dependent and receptor-independent mechanisms are involved. The receptor-dependent mechanisms can play a role at the lower concentrations and receptor- independent at the higher ones.

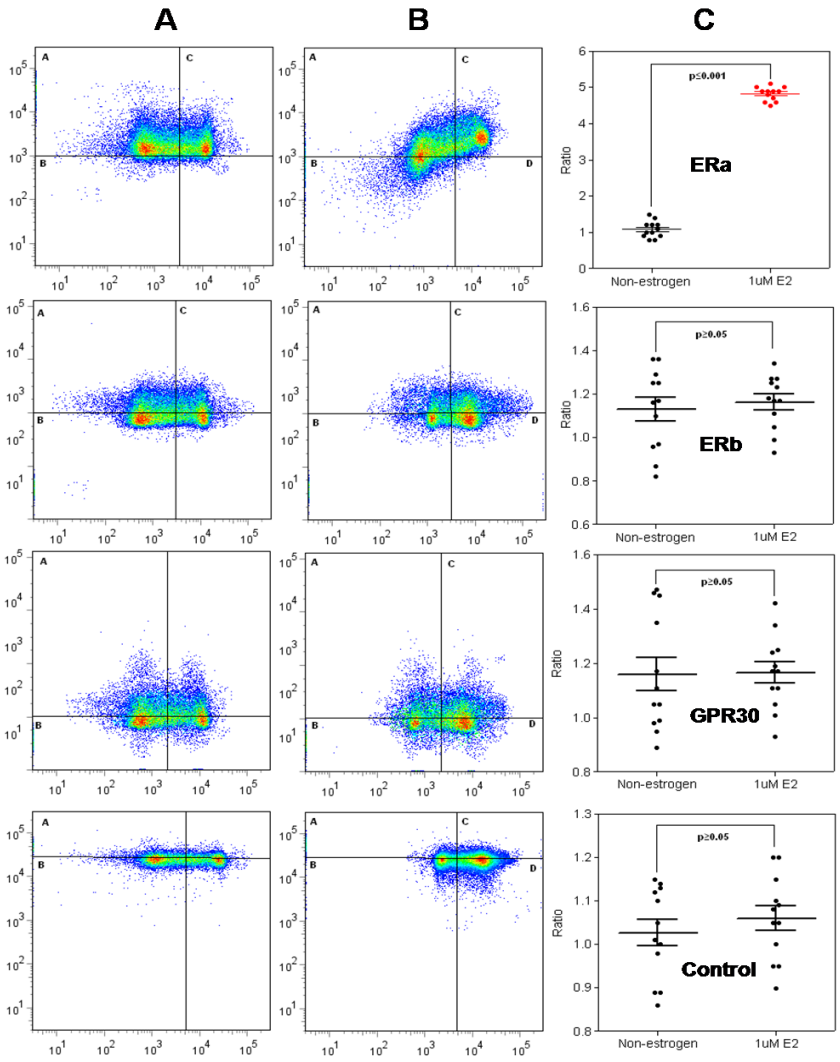

Figure 9. Flow cytometry analysis of boar sperm stained by ACR.2 antibody and antibody against appropriate estrogen receptor in estrogen-free medium (A), the same analysis in the estrogen-enriched medium – 1 μM E2 (B), differences in correlation coefficients between A and B (C).

To determine the role of individual estrogen receptors during estrogen signaling in the sperm, we carried a dual-staining flow-cytometry experiment with antibodies against individual

estrogen receptors and ACR.2, which detect the capacitation progress. We then analyzed the correlation between the individual receptor abundances and capacitation progress detected by ACR.2 in estrogen-free and estrogen-enriched media (Figure 9).

We detected a significant difference between the correlations in estrogen-free and estrogen-enriched media in case of ERα. In case of ERβ we detected no such differences and the signal from the GPR30 detection channel was very weak. We were also unable to detect GPR30 by immunocytochemistry. Contrary to our results, in their current work Rago et al. detected GPR30 in boar and human sperm. Therefore, the determination of the role of membrane-associated type of ERα and GPR30 during estrogen signaling in the sperm will be an issue of our future interest.

4.2. Effect of selected endocrine disruptors on male reproductive parameters and expression of testicular genes (Supplements 4, 5, 6).

In the second major part of our studies, we focused on the effect of environmental pollutants that act as endocrine disruptors on male reproductive parameters in mouse *in vivo* experiments. In our first study (Supplement 4) we tested the effect of two different concentrations (150 μg/l and 0.15 μg/l) of mycotoxin zearalenone (ZEA) on the reproductive parameters and expression of testicular genes in male mice.

We didn't observe any alteration of the body weight and/or weight of reproductive organs between the control and experimental groups. Also, the spermatogenesis and microstructure of testicular tissue were not altered as evaluated by classical histology. However, we found decreased sperm concentration, increased amounts of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexinV. We also performed analysis of gene expression of 28 genes playing important roles in the testicular tissue physiology. In the group exposed to the lower concentration of ZEA (0.15 μg/l) we observed significantly decreased expression of the Vegfa gene, which is specifically expressed in spermatogonial cells. We also detected decreased expression of several genes specific for spermatocytes – Sycp3, Ccna1, and Grth and increased expression of gene Sycp1, which is also expressed in spermatocytes. The expression of genes specific for round spermatids was not changed. In the group exposed to the high dose we did not detect any significant changes in the expression of the tested germ-cell genes. In the case of genes expressed specifically in Sertoli cells, we detected significantly decreased expression of Sox9 genes in both groups and also decreased expression of Wt1 genes in the group exposed to the high dose. In the group exposed to the

low dose we detected increased expression of the Mas1 gene. Two other tested genes (Eps8 and Icap1) did not reveal any significant changes in gene expression. In the case of genes related to epigenetic processes, we detected significantly decreased expression of genes for Ccnd1 and Dnmt1in the animals exposed to the low dose of ZEA and significantly decreased expression of genes for Ccnd1, Kdm4a and Spata2 in animals from the group exposed to the high dose.

In our other study (Supplement 5) we tested the effect of widely used flame retardant tetrabromobisphenol A (TBBPA) on the parameters similar to our ZEA study. We performed two-generational *in vivo* experiment with continuous exposure to 200 μg/l of TBBPA. In the parental generation, control animals were not exposed to TBBPA, and in the experimental group only females were exposed during gestation. In the F1 generation there were two sub-groups – **C** (control group) and **T** (group exposed to TBBPA). In group **T**, the pups were exposed to TBBPA during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood. In group **C**, the pups were not exposed to TBBPA at all. F1 generation animals were bred up to the age of 70 days as follows – mother and father from group **C** formed the group **CC**; both parents from group **T** formed group **TT** (interbreeding), mother from group **C** and father from group **T** formed group **CT** by outcrossing and mother from group **T** and father from group **C** formed group **TC** by reverse outcrossing. Pups of F2 generation were exposed to TBBPA only in groups **TC** and **TT** (group **CC** was not exposed to TBBPA). During body and relative organ weight examination, we observed lower relative testis and prostate weights in the **TT** group. The concentration of TBBPA used in this study had no effect on the sperm parameters and no significant alterations in the testicular tissue morphology and spermatogenesis were observed as well. On the other hand, morphometrical analysis of seminiferous epithelium revealed significantly lower thickness of the seminiferous epithelium in **T**, **TC** and **TT** groups and also a relatively higher number of TUNEL-positive cells in the **T**, **TC** and **TT** groups compared to **C** and **CC** group, respectively. We also observed major changes in the expression of genes involved in the regulation of spermatogenesis and apoptosis in the **TT, TC** and **T** groups.

Finally, in our third study (Supplement 6) we analyzed the effect of TBBPA on the protamine content and DNA integrity in the mouse sperm. In this study we used the same concentration as in our previous TBBPA study (200 μg/l) and exposed the animals during gestation, lactation, pre-pubertal and pubertal periods. We observed a decreased P1/P2 ratio (0.362 vs. 0.494 in controls; $P< 0.001$), increased total protamine/DNA ratio (0.517 vs. 0.324 in controls; $P< 0.001$) and increase in the number of TUNEL-positive spermatozoa (39.5% vs

21.2% in controls; P<0.05). Since protamines play an important role in the sperm epigenome, the differential protaminization of the sperm DNA sequences may represent essential mechanisms enabling trans-generational transmission of the pathological phenotypes induced by EDs and other environmental pollutants.

4.3. Prenatal exposure to endocrine disruptors induces trans-generational deregulation of microRNA expression in male primordial germ cells (Supplement 7).

In the last presented study, we focused on the effect of EDs on the changes in the epigenetic profiles in mouse PGCs in trans-generational experiments. It is known that the effects of EDs vary in relation to the nature of the compounds, dose, and period of exposure during development. Moreover, each ED has its own signature in the gene expression profile, the so called "fingerprint" of the compound, and the effect of EDs on gene expression was disclosed in the previous part of the presented work.

However, the effects of EDs on miRNA expression in PGCs remain undetermined. Therefore, in this study we used the well-known ED vinclozolin to evaluate its effect on microRNA expression profiles in a mouse trans-generational experiment. We exposed pregnant female mice (F0) to two doses of VCZ (VD1, 1 mg/kg/bw/d and VD2, 100 mg/kg/bw/d) and at the 13.5 day of pregnancy we sacrificed part of the animals to analyze embryonic testis and microRNA profiles in PGCs. We used the second part of F0 generation to produce offspring (F1) and males from this generation were used to produce the F2 and subsequently F3 generation. To evaluate whether the exposure to other EDs during the embryonic period could also modify the miRNA expression pattern in PGCs in a compound-specific form, we also performed mouse prenatal exposure to mono-(2-ethylhexyl) phthalate (MEHP) (500 mg/kg/bw/d) and zearalenone (ZEA) (10 mg/kg/bw/d), in addition to VCZ. The exposure to MEHP and ZEA was performed as described above for VCZ. In all experimental groups (VCZ, MEHP and ZEA), we analyzed the expression of microRNA using the microarray technique.

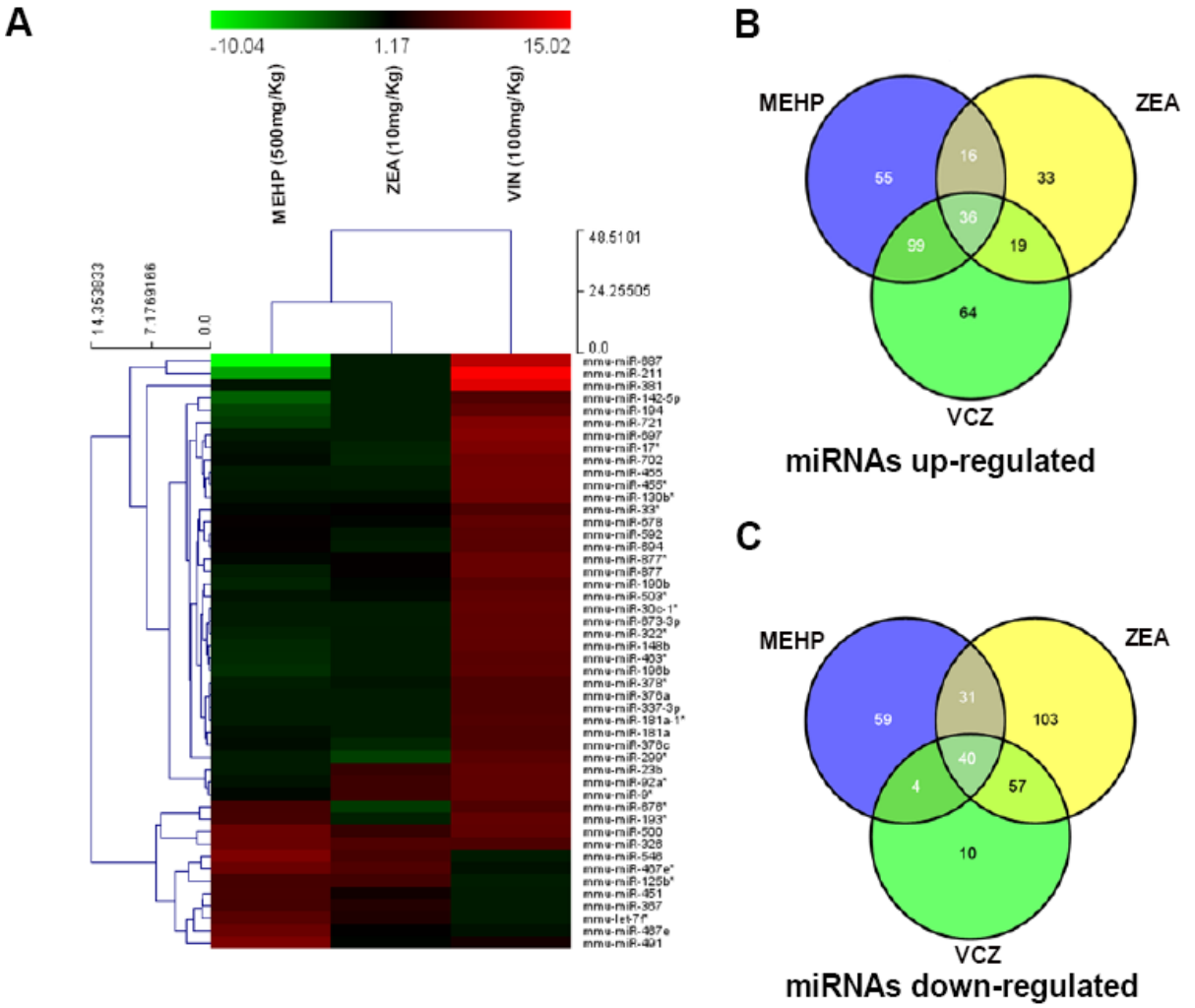

Figure 10. Microarray analysis of the expression of multiple microRNAs among mice exposed to different types of EDs (A), Venn diagrams showing the relations in microRNA expression among individual ED treatments (B, C).

We isolated total RNA from 13.5 dpc F1 exposed PGCs and the levels of 518 mouse miRNAs were quantified by TaqMan rodent miRNA arrays version 2.0 miRNA Megaplex (Applied Biosystems). We observed that exposure to the different compounds altered the miRNA profiles compared to the control (Figure 10.). We observed a common group of 302 miRNA that were deregulated after exposure to the three compounds. Interestingly, exposure to VCZ showed the highest number of up-regulated miRNAs (218). Of them, 29 % were up-regulated only by VCZ (Figure 10B). On the other hand, VCZ was the compound with the lowest number of down-regulated miRNAs (10) (Figure 10C). Of these, 11 % were specifically down-regulated in VCZ-exposed PGCs. Consequently, early embryo exposure to VCZ induced severe and defined alterations in the expression of microRNAs in male PGCs.

Having these results, we wanted to confirm the expression data from microarray by qPCR. We selected VCZ as the first tested compound. Of 136 VCZ-specific deregulated miRNAs,

we selected some of the most representative involved in the PGC fate, survival, differentiation or progression: let-7, miR-21, miR-23b, miR-135, miR-381 and miR-486. We next analyzed their expression patterns from F1 to F3 by qPCR using TaqMan probes. Interestingly, some of the VCZ most affected miRNAs are potential regulators of key genes of PGC specification such as Lin28/Lin28b (let-7, mmu-miR-381, mmu-miR-23b and mmu-miR-21) and Blimp1 (let-7, mmu-miR-23b and mmu-miR-135).

Finally, we also demonstrated deregulation of the expression of Lin28 and Blimp1 at the protein level using the western blot technique.

The remaining question is what mechanisms are responsible for trans-generational transmission of the altered phenotypic and epigenetic patterns. During our analysis we did not observe any major changes in DNA methylation profiles in PGCs and sperm of animals exposed to VCZ. On the other hand, the methodological approach used has its limitations, and it is possible that some important regulatory regions are differentially methylated and this fact could be not uncovered during our analysis. Furthermore, we demonstrated a significant effect of another ED – TBBPA – on the protamination of the sperm DNA and it is possible that the changes in this specific process may also play a role in the case of VCZ. Finally, microRNAs themselves may play a significant role in the trans-generational transmission of the altered phenotypes. The altered microRNA profiles in PGCs can be retained in the spermatogonia and later on in the sperm. After fertilization, the sperm-originated microRNA can alter the methylation of specific DNA sequences via microRNA-dependent DNA methylation. Therefore, the analysis of the microRNA expression patterns in the sperm of exposed animals is another future aim of our work.

5. Conclusion

In presented study, we focused on the effect of estrogens and EDs on various parameters related to the mammalian reproduction in the boar and especially mouse model. According to our results, estrogens, although primarily considered as female hormones, have significant effects on the sperm physiology and these effects can influence the reproductive fitness of the sperm population exposed to different doses of estrogens. Different EDs, and especially those with estrogenic and/or anti-androgenic activity, also significantly alter the expression of genes playing important roles in testicular physiology and spermatogenesis. Finally, we demonstrated a significant, trans-generational effect of selected EDs on the reproductive parameters and epigenetic profiles of PGCs.

In the standard toxicological studies, only a limited number of physiological parameters is measured and analyzed. On the other hand, the tested compounds usually influence multiple aspects of mammalian reproduction. One compound can simultaneously influence the process of sperm capacitation, spermatogenesis and formation of PGCs. Furthermore, in their normal life, outside the controlled experimental conditions, human and wildlife are exposed to hundreds or thousands of organic and synthetic compounds with confirmed or potential effects on mammalian reproduction.

Therefore, the detailed analysis of the different ways by which individual compounds influence and potentially disrupt reproductive processes is an absolute prerequisite for the environmental safety management and also for understanding of the current problems with reproductive health in human population.

We hope that at least some parts of the presented study together with our current work significantly contribute to this issue.

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SUPPLEMENTS

SUPPLEMENT 1

Ded, L, P Dostalova, A Dorosh, K Dvorakova-Hortova, and J Peknicova 2010 Effect of estrogens on boar sperm capacitation in vitro. *Reproductive Biology and Endocrinology* **8**.

My contribution to this work: See “Authors' contributions“ in the supplement.

REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

METHODOLOGY Open Access

Effect of estrogens on boar sperm capacitation *in vitro*

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Abstract

Background: Mammalian sperm must undergo a series of controlled molecular processes in the female reproductive tract called capacitation before they are capable of penetrating and fertilizing the egg. Capacitation, as a complex biological process, is influenced by many molecular factors, among which steroidal hormone estrogens play their role. Estrogens, present in a high concentration in the female reproductive tract are generally considered as primarily female hormones. However, there is increasing evidence of their important impact on male reproductive parameters. The purpose of this study is to investigate the effect of three natural estrogens such as estrone (E1), 17beta-estradiol (E2) and estriol (E3) as well as the synthetical one, 17alpha-ethynylestradiol (EE2) on boar sperm capacitation in vitro.

Methods: Boar sperm were capacitated in vitro in presence of estrogens. Capacitation progress in control and experimental samples was analyzed by flow cytometry with the anti-acrosin monoclonal antibody (ACR.2) at selected times of incubation. Sperm samples were analyzed at 120 min of capacitation by CTC (chlortetracycline) assay, immunocytochemistry and flow cytometry with anti-acrosin ACR.2 antibody. Furthermore, sperm samples and capacitating media were analyzed by immunocytochemistry, ELISA with the ACR.2 antibody, and the acrosin activity assay after induced acrosomal reaction (AR).

Results: Estrogens stimulate sperm capacitation of boar sperm collected from different individuals. The stimulatory effect depends on capacitation time and is highly influenced by differences in the response to estrogens such as E2 by individual animals. Individual estrogens have relatively same effect on capacitation progress. In the boar samples with high estrogen responsiveness, estrogens stimulate the capacitation progress in a concentration-dependent manner. Furthermore, estrogens significantly increase the number of acrosome-reacted sperm after zona pellucida- induced acrosomal reaction.

Conclusions: We demonstrate here the stimulatory effect of four different estrogens on boar sperm capacitation in vitro. According to our results, there is significant difference in the response to tested estrogens at different capacitation time and among individual animals. In animals with a high response to estrogens, there is a concentration-dependent stimulation of capacitation and individual estrogens have relatively the same effect. Effects of individual estrogens, differences in the response to them by individual animals, their time and concentration-dependent outcomes further contribute to our knowledge about steroidal action in sperm.

Background

Capacitation involves the physiological changes that spermatozoa must undergo in the female reproductive tract or *in vitro* to obtain the ability to penetrate and fertilize the egg [1-3]. Capacitation is a complex molecular process that results in changes of calcium concentration, protein phosphorylation, acrosomal matrix and membrane rearrangement. As a complex biological process, capacitation can be influenced by many molecular factors in the uterine and oviductal fluid [4] and the effect of uterine and oviductal fluids depends on the specific stages of the estrous cycle [5]. Although capacitation naturally occurs in the female reproductive tract, it can be also performed *in vitro* using specific media and physical conditions [6,7].

Estrogens are a group of steroid compounds, named for their importance in the estrous cycle. Although estrogens

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have been considered mainly female reproductive hormones, they also play an important role in regulating male reproductive functions. The main breakthrough in this field was brought forth by estrogen receptor knockout mice. Phenotypically, these mice have significant alteration in testes histology, spermiogenesis and they suffer from infertility [8].

In somatic cells, estrogens act through three known estrogen receptors - ERa, ERb and GPR30. ERa and ERb are called - classical estrogen receptor. They bind specific loci in DNA (estrogen response elements) and act as transcriptional factors. Recently, there has been evidence of a nongenomic effect of these receptors [9] and this effect may be important for estrogen regulation of the sperm function since sperm are supposed to be transcriptionally silent. Classical estrogen receptors were found in human spermatozoa and there is evidence for their direct interaction with phosphatidylinositol-3-OHKinase/Akt pathway [10]. This observation is important, because some receptors in sperm membrane are supposed to have only a passive role [11]. Classical estrogen receptors were recently found together with the aromatase and androgen receptor in pig spermatozoa [12]. Beside classical receptors, estrogens can act through the membrane estrogen receptor GPR30. GPR30 signalization is accompanied by calcium mobilization, therefore, a signalization through this receptor seems to be a good candidate for estrogen pathway in sperm. However, to this date there is no evidence for the presence of this receptor in the sperm. Finally, there is some evidence for the presence of putative estrogen receptors in the sperm, which is different from the classical ones. The antibodies against these putative receptors block the stimulatory effect of estrogens but their functions remain to be elucidated [13].

Although several studies report effects of estrogen in mature spermatozoa, there are some contradictory results in this field. There are a few papers from 1970 s - 1980 s concerning the effect of estrogens and progesterone on capacitation of hamster and rabbit sperm *in vivo* and *in vitro*. Gwatkin and Williams reported an inhibitory effect of the follicular fluid enriched by progesterone and estrogens on capacitation of rabbit spermatozoa *in vitro* [14]. Briggs obtained similar results with hamster sperm [15]. Contrary to this, Bathla et al. reported a significantly higher number of spermatozoa incubated in isolated uterus enriched by exogenous estrogens [16]. Further, Hamner and Wilson concluded that antiestrogens have no effect on the capacitation progress of rabbit sperm [17]. Recently, it was reported that there is a stimulatory effect of estrogens and different xenoestrogens on capacitation, acrosome reaction and fertilizing ability of mouse spermatozoa [18]. Furthermore, pre-incubation with estrogens does not alter the ability of human sperm to fuse with the oocyte [19].

In this study, we investigated the effect of three natural estrogens such as estrone (E1), 17β-estradiol (E2), estriol (E3), and one syntetical estrogen (17α-ethynylestradiol, EE2) on capacitation and AR of boar sperm *in vitro*.

Methods

Chemicals

All chemicals were purchased from Sigma (Prague, Czech Republic) unless otherwise specified.

Sperm capacitation *in vitro* and calcium ionophore/zona pellucida-induced acrosomal reaction

Boar (Sus scrofa) ejaculates were supplied by Insemination Station, Kout na Sumave, CR. All sperm samples were examined for their motility and viability. Samples of poor quality were discarded. Suitable sperm samples were washed twice in tris-buffered saline (TBS, 200 × g, 10 min), centrifuged on Percoll gradient (80, 70, 55, 40% Percoll, 200 × g, 60 min) and washed in capacitation medium without bovine serum albumine (11.3 nM NaCl, 0,3 mM KCl, 1 mM $CaCl_2$, 2 mM TRIS, 1.1 mM glucose, 0.5 mM pyruvate). After being washed and percolled, sperm were resuspended in capacitation medium (11.3 nM NaCl, 0.3 mM KCl, 1 mM $CaCl_2$, 2 mM TRIS, 1.1 mM glucose, 0.5 mM pyruvate, BSA 1 mg/ml, pH 7.4) to concentration 5×10^7 sperm/ml. Experimental sperm samples were treated by estrogens to final concentrations 1 nM - 100 μM and control samples with the same amount of ethanol as in the experimental samples. Sperm suspension was incubated for the relevant time (30, 60, 90, 120, 180, 240 min) under paraffin oil at 37°C, 5% CO_2. After 240 min of incubation, selected samples were treated by boar solubilized zona pellucida (ZP) (Czech Univerzity of Life Sciences, Prague, Czech Republic) for 30 min (37°C, 5% CO_2).

CTC assay

The chlortetracycline (CTC) fluorescence assay was described previously [20,21]. After the capacitation process, sperm suspensions were centrifuged: the capacitation medium was removed and refrigerated for biochemical assays. Sperm were re-suspended in phosphate-buffered saline (PBS) and mixed with equal volume (45 μl/45 μl) of CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris-HCl, pH 7.8) and incubated for 30 min. Cells were then fixed by 8 μl of 12.5% paraformaldehyde in 0.5 mol/l Tris-HCl (pH 7.4). After incubation, sperm suspension was placed on a glass slide, smeared and overlaid by a cover slip. To avoid evaporation and CTC fading, slides were kept in a wet chamber until the evaluation was carried out. Samples were examined with a Nikon Labothot-2 fluorescent microscope equipped with a 40× Nikon Plan 40/0.65 and photographed with a COHU 4910 CCD camera (Inc.

Electronics Division, San Diego, USA) with the LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic). Sperm were classified according to their acrosomal staining patterns: (A) Bright fluorescence over the entire sperm head and positive mid-piece of the tail - uncapacitated, acrosome intact sperm; (B) Prominent fluorescent positive equatorial segment, mid-piece of the tail and fluorescence-free (dark) band in the post-acrosomal region - capacitated, acrosome-intact sperm; (C) Low fluorescent signal throughout the sperm head, with remaining positive signal in the equatorial segment and mid-piece - acrosome-reacted sperm (Fig. 1). Sperm with a nonspecific or intermediate fluorescent signal status were not selected for subsequent analysis. In each sample, 200 cells were evaluated and the minimal number of evaluated samples was 5.

Indirect immunofluorescence with anti-acrosin ACR.2 monoclonal antibody

ACR.2 immunofluorescent analysis was described previously [22,23]. After the capacitation process, sperm suspensions were centrifuged; the capacitation medium was removed, and kept at -20°C. Sperm were re-suspended in equal volume of phosphate-buffered saline (PBS), smeared onto glass slides, dried and kept at 4°C. During fluorescent specimen preparation, sperm slides were fixed with acetone for 10 min, rinsed with PBS, treated with ACR.2 monoclonal antibody and incubated in a wet chamber for 60 min at 37°C. After thorough washing in PBS, the smears were treated with FITC-conjugated anti-mouse IgG antibody (Sigma, Prague, Czech Republic) and again incubated in a wet chamber for 60 min at 37°C. After washing in PBS and water, smears were mounted by the Vectashield mounting medium with DAPI (Vector Lab., Burlingame, CA). Samples were examined with a Nikon Labothot-2 fluorescent microscope equipped with 40× Nikon Plan 40/0.65 and photographed with a COHU 4910 CCD camera (Inc. Electronics Division, San Diego, USA) using LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic). Sperm were classified according to their acrosomal staining patterns. (A) Moderate fluorescence in the acrosomal area - uncapacitated, acrosome intact sperm; (B) Intensive fluorescence of the acrosome -- capacitated, acrosome-intact sperm; (C) Low or no fluorescent signal in the sperm head with a remaining positive equatorial segment - acrosome-reacted sperm (Fig. 2). Sperm with nonspecific or intermediate acrosomal status were not selected for subsequent analysis. In each sample, 200 cells were evaluated and the minimal number of evaluated samples was 5.

Flow cytometry analysis with ACR.2 antibody

The control, capacitated and experimental sperm samples from animals with high responsiveness to E (animal A) were influenced by 1 μM E2, then washed in PBS and fixed by 96% ethanol at 4°C for 60 min. After ethanol fixation, sperm were refixed in ethanol-acetone mixture at 4°C (1:1) for 30 min. Sperm were then washed three times in PBS and incubated with anti-acrosin ACR.2 antibody (50 μg/ml) at 37°C for 60 min. After the incubation with the primary antibody, sperm were washed three times in PBS and incubated with a secondary anti-mouse IgG antibody (Sigma, Prague, Czech Republic). After the incubation sperm samples were intensively washed in PBS (five times for 5 min) then 100 μl of the suspension was placed on 96-well plate. Acquisition and analysis were performed on BD LSR II instrument (BD, Becton Drive Franklin Lakes, NJ, USA), excitation laser 488 nm, emission filters 530/40, measurement of fluorescent intensity in FITC channel. Analysis was performed using FlowJo 7.5.4. software (TreeStar Inc., Ashland, OR, USA). The differences among control and experimental samples in arithmetic mean of the fluorescent intensity in the FITC channel were assessed.

Indirect ELISA with ACR.2 antibody

After *in vitro* capacitation, sperm samples were centrifuged and sperm-free capacitating medium was collected

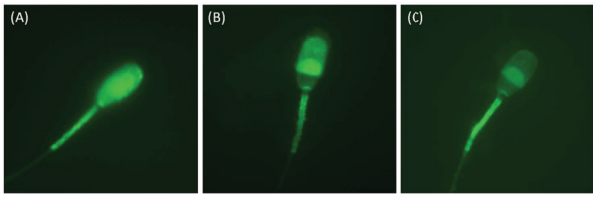

Figure 1 CTC acrosomal fluorescent patterns. Representative pictures of three specific CTC acrosomal fluorescent patterns. (A) Uncapacitated, acrosome intact sperm - bright fluorescence over the entire sperm head and positive mid-piece of the tail; (B) Capacitated, acrosome-intact sperm - prominent fluorescent positive equatorial segment and mid-piece of the tail, fluorescence-free (dark) band in the post-acrosomal region; (C) Acrosome-reacted sperm - low fluorescent signal throughout the sperm head, with a remaining positive signal in the equatorial segment and mid-piece.

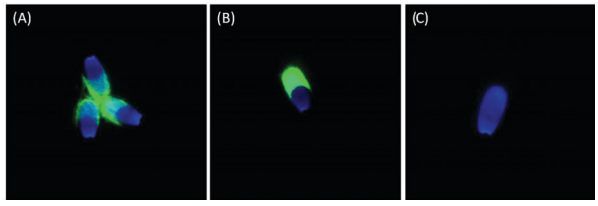

Figure 2 ACR.2 Acrosomal fluorescent patterns (FITC-conjugated secondary antibody). Representative pictures of three specific ACR.2 acrosomal fluorescent patterns. (A) Uncapacitated, acrosome intact sperm - moderate uniform fluorescence in the acrosomal area; (B) Capacitated, acrosome-intact sperm - intensive fluorescence of the acrosome; (C) Acrosome-reacted sperm - low or no fluorescent signal in the sperm head. Nuclei stained with a Blue DAPI dye.

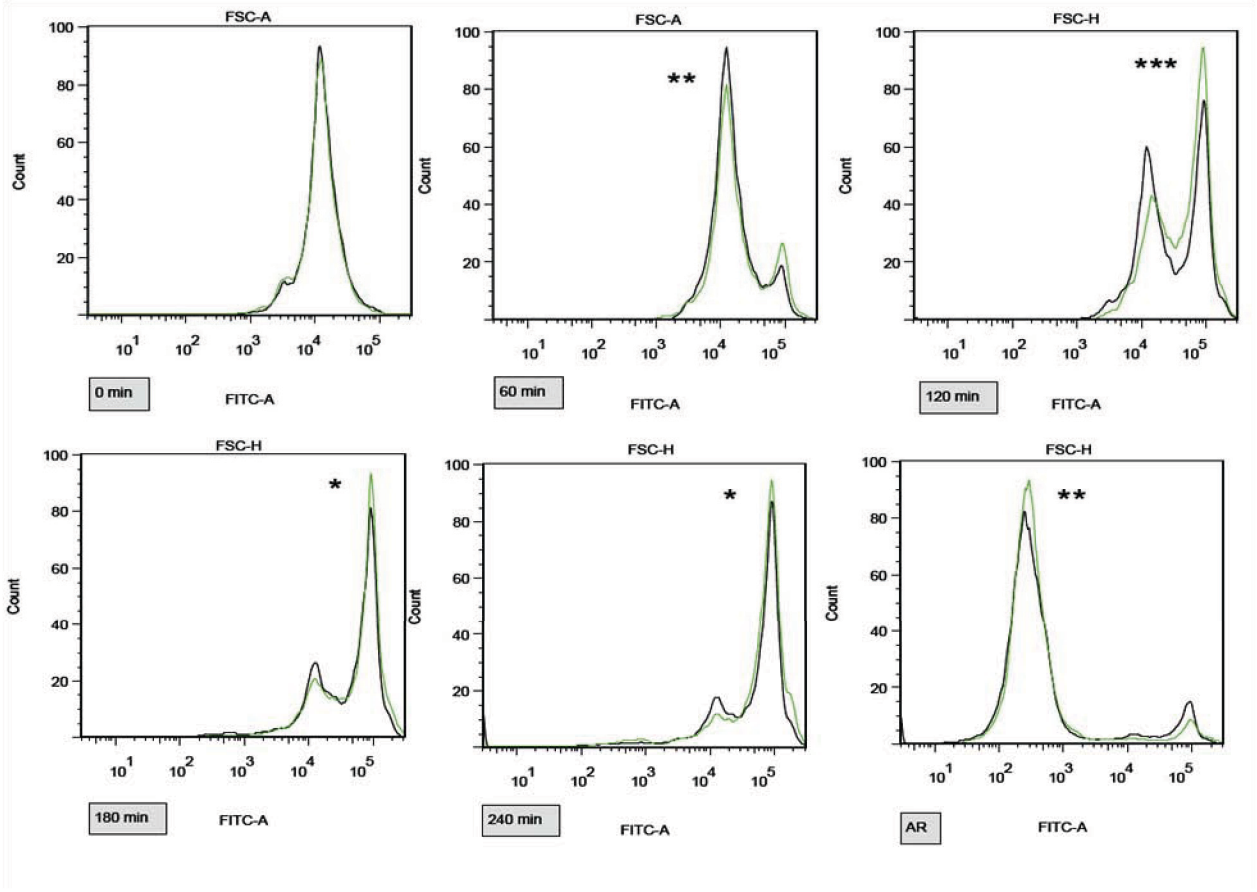

Figure 3 Differences in capacitation and AR progress between samples A-D exposed by 1 μM E2 and control samples measured by flow cytometry with ACR.2 antibody. Representative pictures of FITC channel histograms at 0, 60, 120, 180, 240 min and after induced AR. Control samples in black, experimental samples in green. The increase in fluorescent intensity (right peak) corresponds to the capacitation progress. Differences among the control and experimental samples in arithmetic mean of the fluorescent intensity in the FITC channel were assessed by t-test. *P < 0.05, **P < 0.01, ***P < 0.001. The most significant difference is recognizable at 120 min.

for subsequent biochemical analysis. Capacitation medium was lyophilised and dissolved in determined volume of water. 100 μl of the dissolved lyophilisate was applied on a microtiter plate and incubated for 24 hours. After one-day of incubation, the plate was washed three times by PBS and PBS-TWEEN (2%). The cells were treated by ACR.2 monoclonal antibody [22] and incubated for 60 min. After incubation with primary ACR.2 antibody, the plate was washed and treated with peroxidase-conjugated swine anti-mouse antibody (SWAM-Px, Sevapharma, Prague, Czech Republic) conjugated and incubated for 30 min. After the second incubation, the plate was washed, and cells were treated by *o*-phenylenediamine (Fluka, Buchs, Switzerland) for 3 min. The reaction was stopped by 4N sulfuric acid and the absorbance was measured on Biotrak II Plate Reader (Amersham Biociences) at 492 nm.

Acrosin activity assay

After the *in vitro* capacitation process, sperm samples were centrifuged and sperm-free capacitating medium was collected for subsequent analysis. Capacitation medium was lyophilised and redissolved in 100 μl of reaction buffer (0.2 M Tris.HCl, 0.02 M CaCl2, pH = 8), placed on a microtiter plate and incubated for 10 min. After the first incubation the BAPA solution (1 mg Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride/1 ml dimethylformamide) was added and this was incubated for 20 min. After the second incubation, the reaction was stopped by 30% formic acid and the absorbance of samples was measured on a Biotrak II Plate Reader (Amersham Biociences) at 405 nm.

Statistical analysis

Experimental data were analyzed using STATISTICA 7.0. (StatSoft CR, Prague, Czech Republic). The statistical differences in the number of sperm with specific acrosomal status among control and experimental samples were assessed by the Kruskal-Wallis one-way analysis of variance (KW-ANOVA). Statistical differences between the continuous values (arithmetic means of the fluorescent intensity in the FITC channel in flow cytometry analysis,

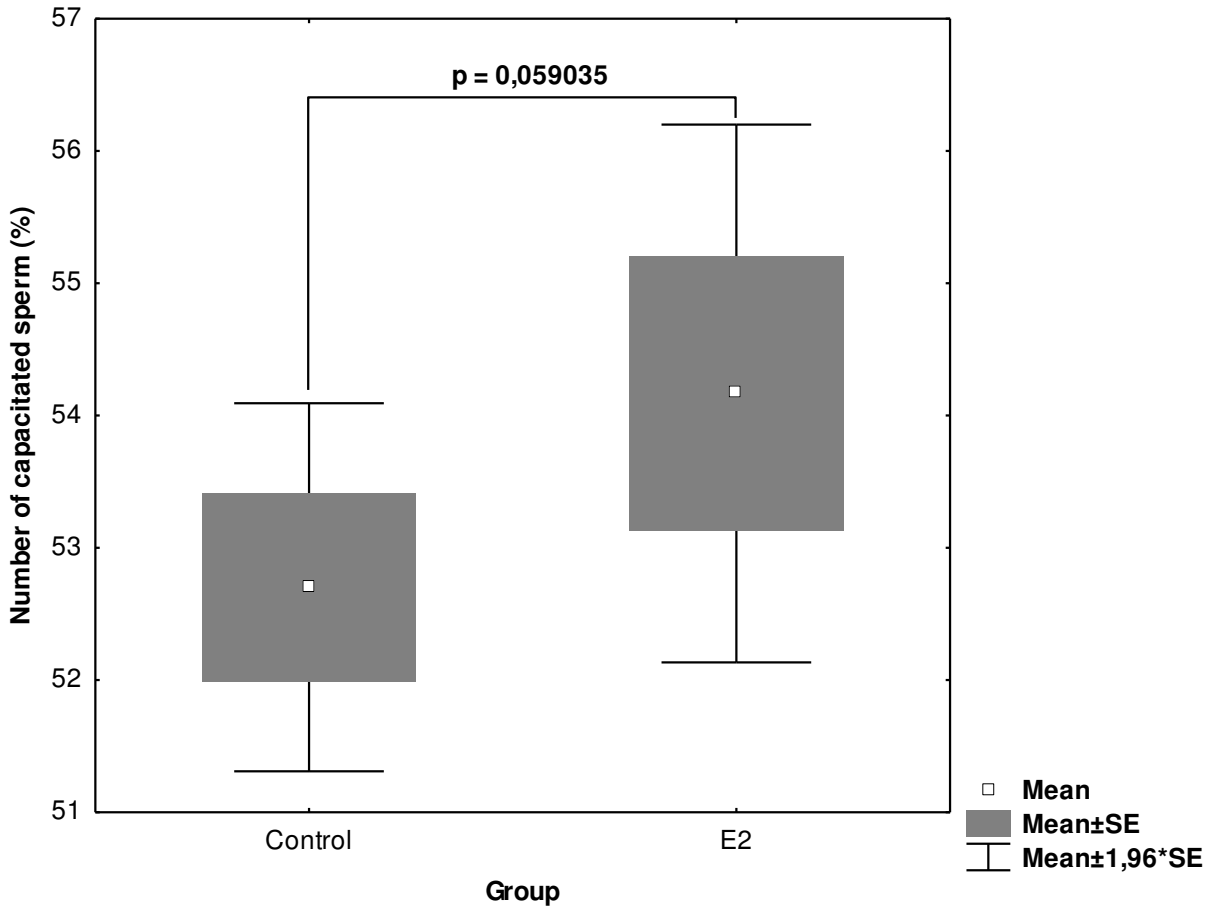

Figure 4 Differences in the number of capacitated sperm in control and experimental samples influenced by 1 μM E2 in 8 individual animals. Sperm samples from 8 individual animals were capacitated with 1 μM E2 and ethanol (control). Sperm were collected after 120 min of capacitation and analyzed by CTC and ACR.2 immunofluorescence. Differences were analyzed by Mann-Whitney U test *P < 0.05, **P < 0.01, ***P < 0.001.

absorbance in indirect ELISA with ACR.2 antibody and acrosin activity assay) were assessed by one-way analysis of variance ANOVA. Post hoc analysis was performed by the Newman-Keuls test and multiple comparisons of mean ranks. The P value, *P < 0.05, **P < 0.01, ***p < 0.001.

Results

ACR.2 flow cytometry analysis of 1 μM E2 effect on capacitation at selected capacitation times

In order to determine the potential differences in the capacitation progress between control and experimental groups, sperm samples were analyzed by flow cytometry with ACR.2 antibody at selected times of capacitation. The experimental sample was capacitated with E2 at a 1 μM concentration, and the control sample with the same amount of ethanol as in the experimental sample. 1 μM concentration of E2 was selected based on the previous mouse study [18] where it was defined as the lowest concentration with any significant effect on sperm capacitation. Sperm were collected at 0, 10, 30, 60, 120, 180, and 240 min of capacitation and after the induced acrosomal reaction. The first significant difference between the control and experimental group was at 60 min capacitation in the arithmetic mean of the fluorescent intensity in the FITC channel (Fig. 3). The strongest significant difference was then at 120 min of capacitation. After an induced acrosomal reaction, a significantly higher number of sperm underwent AR in the experimental group in comparison with the control one.

Analysis of 1 μM E2 effect on capacitation by CTC fluorescence assay and anti-acrosin ACR.2 monoclonal antibody

Sperm samples from 8 animals were capacitated in parallel, in the presence of experimental concentration of 1 μM E2 and ethanol (control) collected after 120 min of capacitation and analyzed by CTC and ACR.2 immunofluorescence. Only highly correlated results were used in the

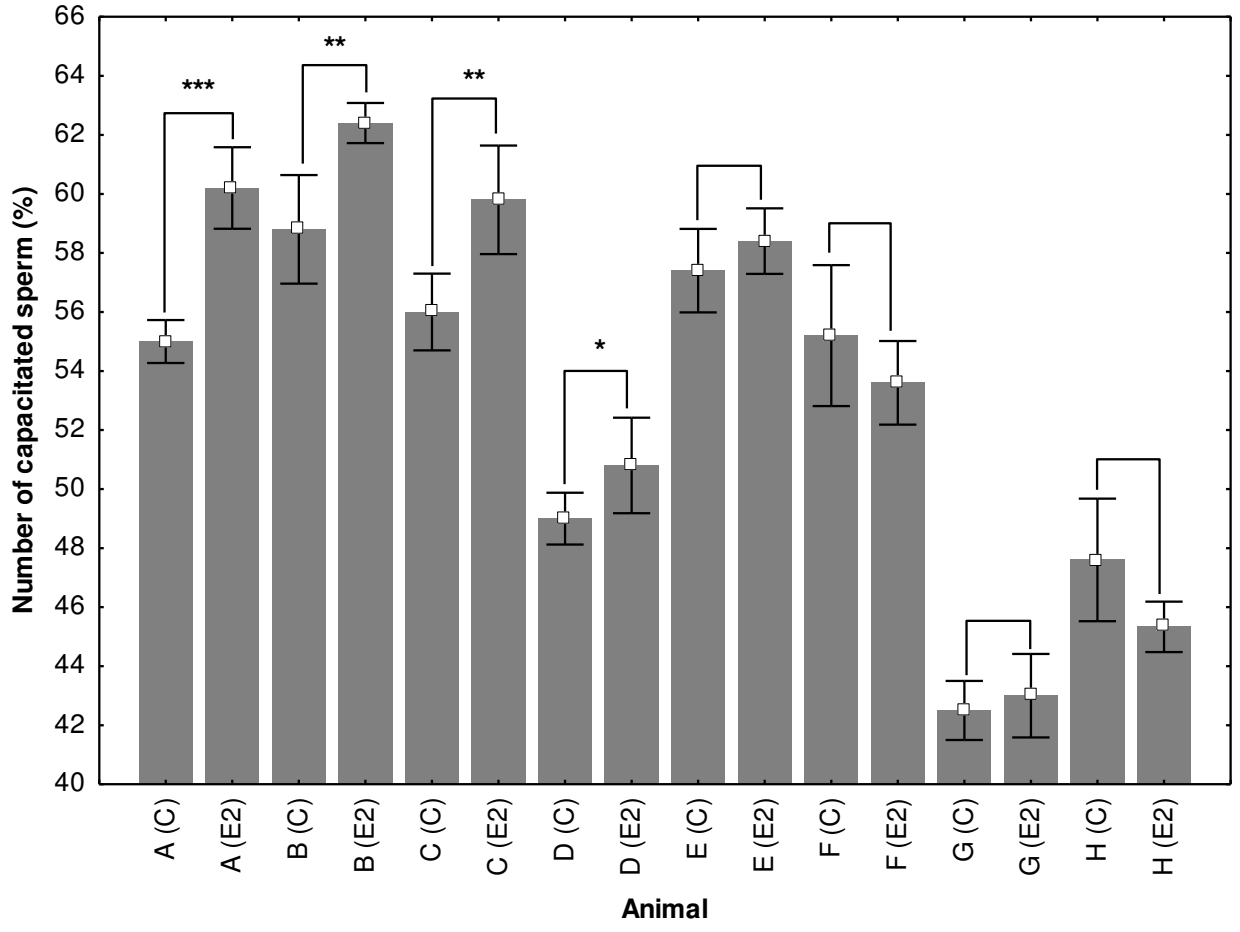

Figure 5 Differences in the capacitation progress and response to estrogen (E2) among individual animals. Animals A-H. Control samples (A (C) - H (C) and samples incubated with 1 μM E2 (A (E2) - H (E2). Whiskers denote ± SE. Differences were analyzed by Mann-Whitney U test, *P < 0.05, **P < 0.01, ***P < 0.001.

subsequent statistical analysis. E2 has almost significant (p = 0.059) procapacitation effect on boar sperm (Fig. 4). In order to evaluate the potential differences in the capacitation progress and the responsiveness to E2 among individual animals the samples from each boar were analyzed separately. There were significant differences in the capacitation progress and responsiveness to estrogens among individual animals (Fig. 5). In 4 samples, E2 significantly increased the number of capacitated sperm; in 4 other samples, E2 had no significant effect on the capacitation progress.

CTC and ACR.2 analysis of the different estrogen-concentration effect at 120 min capacitation in samples with high response to E2 (boar A) and no significant response to 1 μM E2 (boar E)

Since the most significant difference between the control and E2-influenced experimental group was at 120 min of capacitation, sperm samples from boar A (with a high responsiveness to E2) and from boar E (with no significant response to 1 μM E2) were capacitated with a different concentration of four estrogens and analyzed by CTC and immunocytochemistry with ACR.2. In each group, at least 5 samples were analyzed. In CTC assay and immunocytochemistry with ACR.2 antibody, only highly correlated results (difference < 5%) were used in the subsequent statistical analysis. In the boar A sample, all estrogens significantly accelerate the capacitation progress in a concentration-dependent manner (Tab. 1). E2 showed the first significant effect at 10 nM concentration. All selected estrogens significantly accelerate the capacitation progress at 100 nM concentration. In sperm samples from boar E, only high concentrations of estrogens (10-100 μM) stimulated the capacitation progress (Tab. 2).

Analysis of the differences in the number of sperm after ZP-induced AR incubated with 1 μM estrogens

Sperm samples were analyzed after 240 min of capacitation and induced AR by CTC, immunocytochemistry and

Table 1: Number of capacitated sperm in control and experimental samples after 120 of capacitation

Group	Control	1 nM	5 nM	10 nM	100 nM	1 μM	10 μM
E1	55.00 ± 1.56	55.5 ± 0.93	55.45 ± 1.79	56.25 ± 1.28	**57.60 ± 1.26****	**59.75 ± 1.58*****	**61.50 ± 0.93*****
E2	55.00 ± 1.56	56.00 ± 1.41	**56.74 ± 1.21***	**56.80 ± 1.62***	**57.73 ± 1.27*****	**60.20 ± 1.93*****	**62.00 ± 1.41*****
E3	55.00 ± 1.56	54.88 ± 1.36	55.14 ± 1.75	56.14 ± 1.35	**57.00 ± 0.89****	**59.78 ± 1.48*****	**61.71 ± 0.76*****
EE2	55.00 ± 1.56	55.44 ± 1.81	54.99 ± 2.25	55.80 ± 1.93	**56.82 ± 2.04***	**58.30 ± 2.91****	**62.00 ± 1.41*****

Sperm samples from boar A (with high responsiveness to E2) were capacitated in the presence of six different concentrations (1 nM - 10 μM) of four estrogens and analyzed by CTC and immunocytochemistry with ACR.2. In each group, at least 5 samples were analyzed. In CTC assay and immunocytochemistry with ACR.2 antibody, only highly correlated results (difference < 5%) were used in the subsequent statistical analysis. All estrogens significantly accelerated the capacitation progress in a concentration-dependent manner. E2 had the first significant effect at 10 nM concentration, other three estrogens at 100 nM concentration. Differences were analyzed by KW-ANOVA; post hoc comparison was performed by multiple comparisons of mean ranks. *P < 0.05, **P < 0.01, ***P < 0.001.

ELISA with ACR.2 antibody. The acrosin assay was used to further evaluate the effect of estrogens on capacitation and the acrosomal reaction. There was a significantly higher number of sperm, which underwent ZP -induced AR in all experimental samples in comparison with the experimental group (Fig. 6). Data from immunocytochemistry were further verified by ELISA with ACR.2 antibody and the acrosin assay (Fig. 7).

Discussion

In this study, we addressed several questions concerning the effect of estrogens on boar sperm *in vitro*. Although several previous studies have reported on the effects of estrogen in mature spermatozoa among different species, there are some contradictory results in this field. Therefore we employed multiple evaluation techniques to complexly analyze the effect of estrogen on boar sperm *in vitro*. The obtained results from each method might be useful whilst searching for specific mechanisms, which mediate the estrogen effect in mammalian sperm.

The first experiment addressed the question of whether strong, naturally occurring estrogen (E2) has a significant impact on the boar sperm capacitation progress at different capacitation times. Sperm were capacitated *in vitro* in the presence of 1 μM E2 or ethanol (control). We found out that 1 μM E2 has a procapacitation effect on the boar sperm *in vitro*. Furthermore, we demonstrated the non-identical effect of E2 on capacitation at different times of incubation. The first significant difference between the control and experimental samples was at 60 min, and the strongest response was at 120 min of capacitation. In the later capacitation stages 180 min onwards, the difference between the control and experimental samples was not significant. The observed time-dependent effect of estrogens on the capacitation process is an important finding. In previous publications, authors analysed sperm capacitation status after 30 min [18], 180 - 300 min [16] and 360 min [14] and this fact might be an important source for some of the contradictory results. Therefore, the effect of estrogens on capacitation of mammalian sperm should be analyzed at carefully selected capacitation times reflecting the status of the ongoing sperm capacitation process in individual species. Furthermore, the analysis of the time-dependent effect of estrogens on capacitation might be useful while searching for specific molecular processes, which are temporally correlated with the most sig-

Table 2: Number of capacitated sperm in control and experimental samples after 120 of capacitation

Group	Control	1 nM	10 nM	100 nM	1 μM	10 μM	100 μM
E1	57.4 ± 1.14	57.5 ± 1.89	57.55 ± 1.78	57.37 ± 2.62	58.11 ± 1.58	**60.12 ± 1.12****	**61.25 ± 1.56*****
E2	57.4 ± 1.14	58.00 ± 1.61	58.80 ± 1.62	58.11 ± 2.11	58.40 ± 0.89	**60.10 ± 1.22****	**61.81 ± 1.22*****
E3	57.4 ± 1.14	56.89 ± 1.75	56.14 ± 1.35	57.00 ± 1.76	57.78 ± 2.45	**60.22 ± 1.56****	**61.54 ± 1.67*****
EE2	57.4 ± 1.14	57.84 ± 2.09	57.70 ± 1.93	58.42 ± 1.36	58.30 ± 1.98	**60.10 ± 2.31****	**61.43 ± 1.37*****

Sperm samples from boar E (with no significant responsiveness to E2 at concentration of 1 μM) were capacitated in the presence of six different concentrations (1 nM - 100 μM) of four estrogens and analyzed by CTC and immunocytochemistry with ACR.2. In each group, at least 5 samples were analyzed. In CTC assay and immunocytochemistry with ACR.2 antibody, only highly correlated results (difference < 5%) were used in the subsequent statistical analysis. All estrogens significantly accelerated the capacitation progress at 10 μM concentration. Differences were analyzed by KW-ANOVA; post hoc comparison was performed by multiple comparisons of mean ranks. *P < 0.05, **P < 0.01, ***P < 0.001.

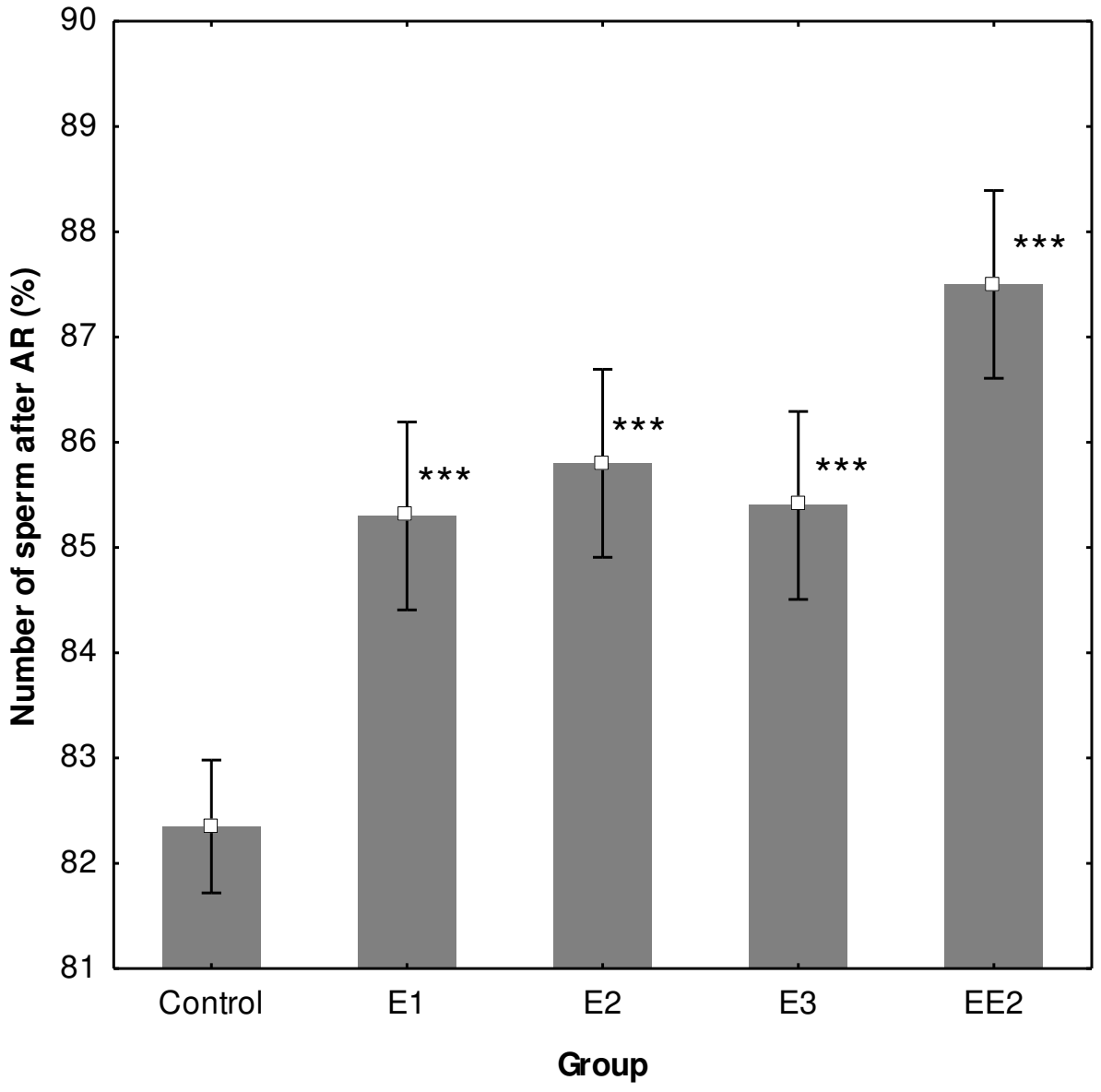

Figure 6 Number of sperm after AR evaluated by ACR.2 antibody. After 240 min of capacitation, control and experimental samples from boars A-D with 1 μM concentration of four estrogens were treated by zona pellucida to induce AR. There was a significantly higher number of sperm, which underwent calcium ZP-induced AR in all experimental samples in comparison to the experimental group. All estrogens significantly increased the number of sperm after the ZP-AR. Differences were analyzed by KW-ANOVA; post hoc comparison was performed by multiple comparisons of mean ranks. Whiskers denote ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.

nificant effect of estrogens (e.g. calcium influx, cholesterol efflux, actin polymerisation, protein phosphorylation, acrosomal rearrangement etc.) [24].

In the second experiment, we wondered whether E2 has a similar effect on sperm samples collected from different individuals. We observed strong differences in the response to estrogens among samples from different individual animals during capacitation *in vitro*. According to our results, the analysis of different responsiveness to estrogens among individual animals in the tested population might be important, because individual variability strongly affects general results. Furthermore, a detailed analysis of the individuals with high and low estrogen-responsiveness can elucidate the mechanism of the estrogen action in sperm. Hitherto, there is no plausible parameter e.g. expression of a different estrogen receptor correlating with estrogen responsiveness [25].

In the third experiment, we tested the effect of four different estrogens on the capacitation progress of sperm collected from boar with high and no significant differ-

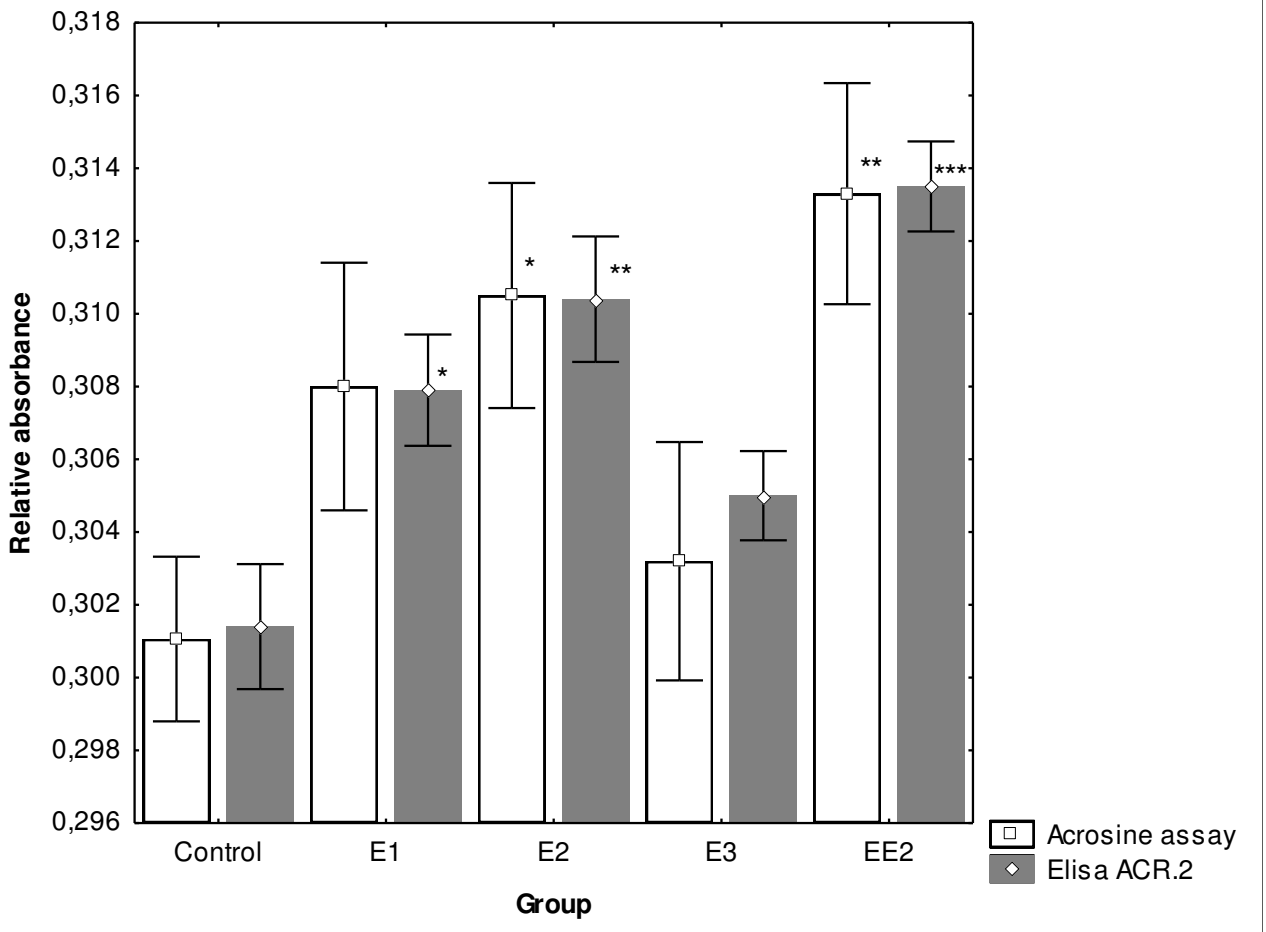

Figure 7 Concentration of acrosin in medium after induced AR measured enzymatically by acrosin assay and immunochemically by ELISA with ACR.2 antibody. After 240 min of the *in vitro* capacitation process, experimental samples from boars A-D with 1 μM concentration of four estrogens were treated by boar zona pellucida to induce AR. Capacitation media were analyzed by ELISA with ACR.2 antibody and acrosin assay to biochemically determine the number of sperm after AR. Differences were analyzed by ANOVA; post hoc comparison was performed by Newman-Keuls test. Whiskers denote ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.

ence. Analysis of the effect of multiple compounds with a similar physiological effect (E1, E2, E3, EE2) provides more reliable data than analysis based only on one compound. In an animal with high estrogen-responsiveness, estrogens stimulate capacitation in a concentration dependent manner. E2 has a significant effect at 5 nM concentration; all other estrogens have a significant effect at 100 nM concentration. Although contrary to other estrogens, E2 had a significant effect at 5 nM and 10 nM concentrations. There was no significant difference between individual estrogens at the appropriate concentration level. In the boar sample with no significant response to 1 μM E2, only a very high concentration of estrogens stimulates capacitation (10-100 μM). It suggests that estrogens have a general procapacitation effect, but in some animals, the responsiveness to estrogens is low and only very high concentrations of estrogens are able to provoke a procapacitation effect. The differences in estrogen-responsiveness further suggest that multiple mechanisms in the estrogen action in sperm might be involved. In the samples with high responsiveness, estrogens (E2) have a significant effect at concentrations normally required for estrogen receptor-mediated cellular response [26,27]. The fact that a higher concentration of estrogens at which estrogen receptors are almost saturated will still increase the number of capacitated cells in a concentration-dependent manner suggests that the estrogen effect at high concentration might be mediated by another, nonreceptor mechanism (membrane changes, etc.) [25]. This idea is further supported by the fact that in samples with no significant response to 1 μM E2, estrogens have a significant effect at high concentrations (10-100 μM), which are far from a concentration needed for the estrogen receptor mediated cellular response in somatic cells. This fact suggests that the specific mechanism (e.g. receptor signalization), which is responsible for

estrogen responsiveness at low concentration, is not functional in samples with no response to 1-10 nM E2. Nevertheless, high experimental concentrations of estrogens (10-100 μM) are far from the physiological plasma levels of estrogens (e.g. 10^{-10}-10^{-11}M for E2 in rats and mice [28]). However, the concentration of estrogens in follicular fluid is on the other hand higher [28] and sperm may be, therefore, exposed to high concentrations during their capacitation in the female reproductive tract [29,30].

Finally, in the last experiment, we demonstrated the significant impact of estrogens on the ZP-induced acrosomal reaction. The number of sperm after AR was significantly higher in all experimental groups. The induced acrosomal reaction data were evaluated microscopically and also confirmed by objective biochemical methods. Therefore, the results obtained from ZP-induced acrosomal reaction, not only confirm the capacitation experiment, but also suggest that estrogens have a real physiological impact on sperm capacitation, as the analysis was based, in particular, on molecular and cellular markers of capacitation (calcium influx, acrosomal rearrangement). Furthermore, the analysis by objective biochemical methods (ELISA, acrosin assay) provides an important supporting data to the subjective microscopical evaluation methods.

In conclusion, in this study we addressed several important questions concerning the effect of estrogens on boar sperm capacitation *in vitro*. We found out that in boar sperm *in vitro* estrogens generally show a procapacitation effect. This effect depends strongly on the stage of the capacitation progress, estrogen concentration and individual responsiveness of tested animals. Individual estrogens have a relatively similar effect. These observations have a significant impact on our understanding of the previous results concerning estrogen effects in sperm and should be helpful to uncover the specific mechanisms of the estrogen effects in sperm physiology.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LD is responsible for sperm capacitation, analysis of the capacitation process by all described methods, for statistical analysis and preparation of the manuscript. PD is responsible for the preparation of the media with specific concentrations of estrogens, sperm capacitation, analysis of sperm capacitation status by CTC and immunocytochemistry by ACR.2 antibody. AD is responsible for sperm capacitation and analysis of capacitation media after the induced AR. JP and KH are responsible for the conception of the study, coordination of experiments, revising and final approval of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENT 2

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My contribution to this work: I performed statistical analysis of the data, contributed to the interpretation of the results and helped to prepare the manuscript.

REPRODUCTION
RESEARCH

The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens

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Abstract

In order for mammalian sperm to obtain a fertilizing ability, they must undergo a complex of molecular changes, called capacitation. During capacitation, steroidal compounds can exert a fast nongenomic response in sperm through their interaction with plasma membrane receptors, and activate crucial signaling pathways leading to time-dependent protein tyrosine phosphorylation (TyrP). Estrogen receptor beta was detected in epididymal mouse sperm; therefore, the effect of 17B-estradiol, estrone, estriol, and 17A-ethynylestradiol on mouse sperm capacitation *in vitro* was investigated. The effect was evaluated by positive TyrP in sperm heads and in the whole sperm lysates. Simultaneously, the state of the acrosome after the calcium ionophore-induced acrosome reaction was assessed. Generally, estrogens displayed a time and concentration-dependent stimulatory effect on sperm TyrP during capacitation. In contrast, the number of sperm that underwent the acrosome reaction was lower in the experimental groups. It has been demonstrated that both natural and synthetic estrogens can modify the physiological progress of mouse sperm capacitation. The potential risk in the procapacitation effect of estrogens can also be seen in the decreased ability of sperm to undergo the acrosome reaction. In conclusion, the capacitating ability of sperm can be significantly lowered by increasing the level of estrogens in the environment.

Reproduction (2011) **143** 1–11

Introduction

The ejaculated mammalian sperm are unable to fertilize the egg. The spermatozoa must undergo a maturation process that is known as capacitation, and it takes place in the female reproductive tract. Only capacitated sperm are able to successfully fertilize the egg (Austin 1952). These morphological and biochemical changes allow sperm to bind to egg zona pellucida and undergo the acrosome reaction (Yanagimachi 1994). One of the processes triggering capacitation is a spontaneous efflux of cholesterol from the sperm plasma membrane. This increases its permeability and fluidity and activates intracellular signalization pathways leading to the activation of adenyl cyclase, cAMP, cAMP-dependent protein kinase A, protein tyrosine kinase, and consequently to protein tyrosine phosphorylation (TyrP; Visconti *et al*. 1995*b*). The TyrP of proteins with a molecular weight (MW) between 40 and 120 kDa became an indicator of successfully ongoing capacitation and the ability of sperm to undergo the acrosome reaction (Visconti *et al*. 1995*a*). The other initiating process leading to the capacitation and acrosome reaction (AR) is the binding of estrogens onto estrogen receptors (ERs).

Estrogens play an important role in both the female and male reproductive systems (Free & Jaffe 1979). They regulate cell function through ERs (ERA and ERBβ; Lubahn *et al*. 1993), which belong to a superfamily of ligand-activated transcription factors (Evans *et al*. 1987) and they are localized in the cytoplasm and nucleus of somatic and germinal cells. The nuclear receptors activate gene transcription by binding to DNA regulatory sequences (Hall *et al*. 2001). However, many nuclear receptors are translocated to the sperm plasma membrane and facilitate not only the gene transcription but also the rapid nongenomic signalization pathways (Pedram *et al*. 2007). Beside these, in sperm G protein-coupled receptors such as GPR30 are reported that trigger not only a rapid nongenomic signalization but also act independently of ERs (Filardo & Thomas 2005). Estrogens are mediated through rapid intracellular signaling in sperm by tyrosine and ser/thr kinases, influencing capacitation and acrosome reaction by activating phosphorylation of several proteins (Naz & Rajesh 2004). Estrogens activate ERs and consequently initiate several intracellular signaling enzymes such as receptor tyrosine kinase, epidermal growth factor receptor phosphatidylinositol 3-kinase, Src kinases,

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mitogen-activated protein kinase (MAPK), protein kinase C, phospholipase D, and phospholipase C. This rapid pathway through MAPK is required for regulating protein phosphorylation in sperm (Aquila *et al.* 2004). Epididymal sperm are believed to have no transcription activity; therefore, a rapid nongenomic signalization plays the main role in this stage.

The concentration of estrogens quantitatively differs between males and females, and it is species specific, as well as the expression of steroid receptors. In males, the concentration of estrogens in the blood plasma is in a range of 2–180 pg/ml and depends on the species. In rat and mouse, the concentration of 17B-estradiol (E_2) is 2–25 pg/ml, and it is lower than in that rete testis fluid (250 pg/ml). The same applies to females where estrogen concentration in the ovarian fluid is at least twofold higher compared with plasma (Free & Jaffe 1979, Hess *et al.* 1995), and in rat and mouse it fluctuates during the estrus, e.g. for E_2 between 145 and 2100 pg/ml (Shaikh 1971). Besides physiologically endogenous estrogens, organisms including humans are exposed to environmental estrogens, which can display false hormone like activity. It is striking that the wastewater treatment technologies are not efficient enough to prevent a further contamination of surface water supplies (Kusk *et al.* 2011). There is enough evidence in literature showing a positive correlation between rising concentrations of environmental estrogens and increasing reproductive abnormalities (Storgaard *et al.* 2006) even their extremely low concentrations (ng/ml) can have an adverse effect on reproduction.

In this study, we localized ERB in mature mouse spermatozoa from cauda epididymis. In consequence to this finding, we aimed to look in detail, whether, selected estrogens (E_2, estrone, estriol (E_3), and 17A-ethynylestradiol) can influence sperm ability to capacitate and undergo induced acrosome reaction *in vitro*. Monitoring of TyrP of specific proteins (MW 40–120 kDa) in the mouse sperm head and tail was taken as a marker of successfully ongoing capacitation reflecting male reproductive fitness.

Results

ERB is present in the epididymal mouse spermatozoa

As shown in Fig. 1, ERB was detected by chemiluminescence in a whole sperm lysate obtained from cauda epididymis. A single band was marked on a nitrocellulose membrane corresponding to a MW of 64 kDa. ERB was also detected by immunofluorescent labeling in fixed mouse spermatozoa from distal regions of cauda epididymis. ERB was clearly localized as a thin sickle over the apical acrosomal region of the sperm head protruding as far as the apical hook (Fig. 2A). The staining pattern was markedly speckled and uniformly present in all intact spermatozoa.

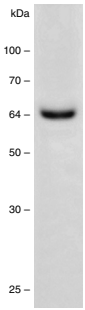


Figure 1 SDS–PAGE and western blotting immunoprotein detection of ERB in a whole sperm cell sample corresponding to a band of molecular weight 64 kDa. The sample contained a protein equivalent of 10^6 cells. Representative result shown.

Estrogens increase protein TyrP in the sperm head during capacitation in vitro

The positive staining for TyrP in epididymal mouse sperm was detected as a compact signal over the whole apical acrosomal region of the sperm head. There was also invariable positive labeling detected in the sperm tail, localized in clusters in the mid and principal piece (Fig. 2B). All sperm that displayed positive head TyrP displayed always a positive tail phosphorylation staining as well.

In general, using immunofluorescent labeling, there was a dose-dependent increase in the number of sperm,

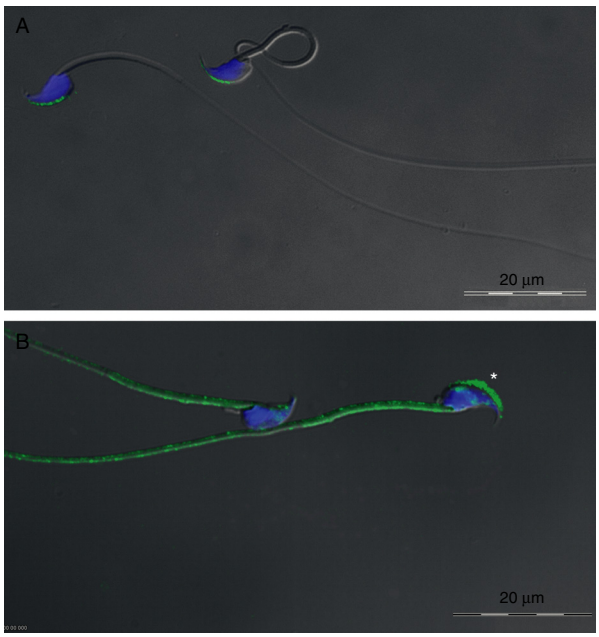


Figure 2 Immunofluorescent detection of ERB of tyrosine phosphorylation in mouse spermatozoa. (A) Immunostaining for ERB (green) over the apical acrosomal region and apical hook of the sperm head. (B) Tyrosine phosphorylation (green) over the apical acrosomal region of the sperm head and in the mid and principal piece of the sperm tail. Positive sperm head labeling showed by asterisk. Nuclei are counterstained with DAPI (blue). Scale bar represents 20 µm.

positive for TyrP in the sperm head during capacitation *in vitro* in the presence of E_2, estrone, E_3, and 17A-ethynylestradiol (Table 1). Results are shown for E_2, as a linear time kinetics (Fig. 3A), and a log concentration kinetics (Fig. 3B) of positive sperm head TyrP during capacitation.

The sperm head TyrP increase in the presence of all estrogens occurred already at 30 min of capacitation (Fig. 3C) and carried to over 60 and 90 min, or even up to 120 min in case of 17A-ethynylestradiol (Table 1). In comparison with control, the two highest concentrations (20 and 200 ng/ml) of E_2, estrone, and 17A-ethynylestradiol significantly increased the protein TyrP compared to E_3, which was effective only at lower doses (0.02, 0.02, and 2 ng/ml; Table 1; Supplementary Figure 2C and D, see section on supplementary data given at the end of this article). Based on selected concentrations in presence of E_2 (Fig. 3A and B) and estrone (Supplementary Figure 1A and B, see section on supplementary data given at the end of this article), there was an increased activity of sperm signaling pathways leading to phosphorylation of specific sperm head proteins during capacitation in higher concentrations (0.2, 2, 20, and 200 ng/ml). Interestingly, compared to the natural estrogens, the stimulatory effect of synthetic 17A-ethynylestradiol on protein TyrP lasted until 120 min of capacitation, especially in the presence of the two highest concentrations (20 and 200 ng/ml; Supplementary Figure 1E and F).

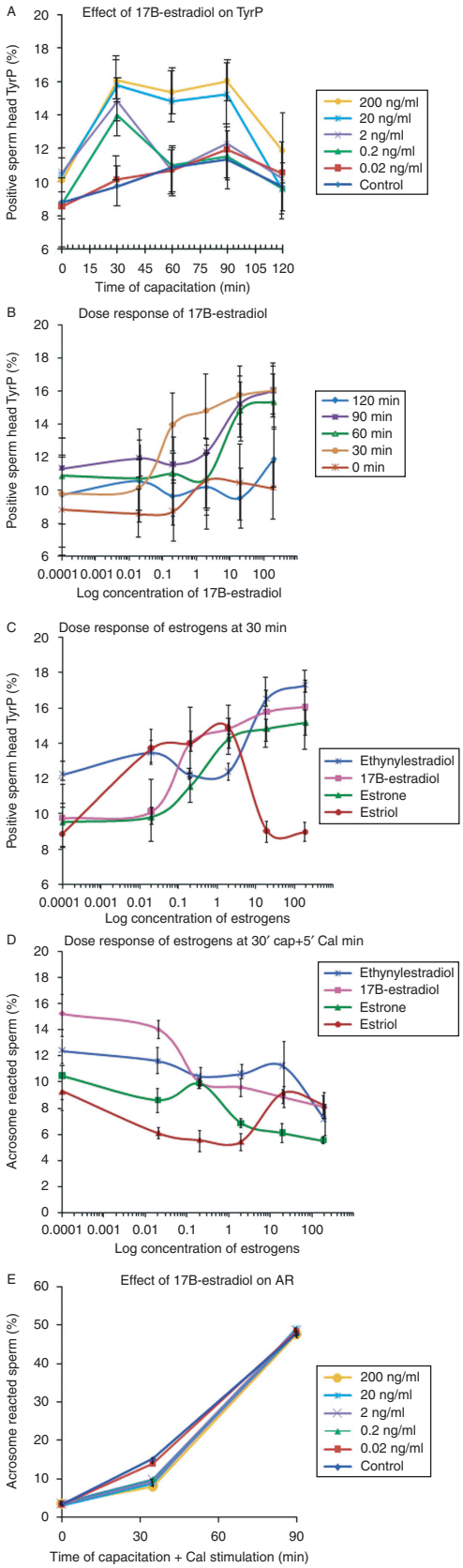
Estrogens decrease the number of acrosome-reacted sperm after calcium ionophore-induced acrosome reaction

E_2, estrone, and E_3 displayed a similar effect on calcium ionophore (CaI)-induced acrosome reaction. These natural estrogens significantly reduced the number of acrosome-reacted mouse sperm *in vitro* (Table 2). The effect was prominent in those sperm that were capacitated for 30 min and then incubated with CaI for only 5 min, however, the effect differed among estrogen concentrations (Fig. 3D). E_2 and estrone significantly decreased the number of acrosome-reacted sperm particularly in the three highest concentrations of 2, 20, and 200 ng/ml (Fig. 3E, Supplementary Figure 2A). Moreover, in the case of E_2 the effect was also statistically significant for 0.2 ng/ml. A similar effect was observed in the case of E_3, in whose presence, there was a statistically significant decrease in the number of acrosome-reacted sperm in the three lowest concentrations, 0.02, 0.2, and 2 ng/ml. The effect remained for the E_3 concentration of 0.2 ng/ml in a group of sperm,

Table 1 Effect of estrogens on protein tyrosine phosphorylation in mouse sperm head during capacitation.

	Time of capacitation (min)				
Concentration (ng/ml)	0	30	60	90	120
17B-estradiol					
Control	8.797±3.038	9.744±1.772	10.856±1.634	11.322±1.793	9.701±2.275
0.02	8.544±1.432	10.121±1.815	10.710±1.929	11.931±1.811	10.533±1.991
0.2	8.714±1.519	**13.956±1.507**‡	11.015±1.923	11.554±1.325	9.633±2.005
2	10.568±2.224	**14.814±1.664**‡	10.715±1.937	12.298±1.261	10.201±2.148
20	10.431±2.262	**15.769±1.697**‡	**14.834±1.772**‡	**15.229±1.656**‡	9.519±1.996
200	10.106±1.883	**16.062±1.595**‡	**15.371±1.718**‡	**16.051±1.331**‡	11.866±1.889
Estrone					
Control	9.429±0.474	9.527±0.516	10.376±0.492	11.932±0.507	10.829±0.534
0.02	9.196±0.542	9.816±0.646	11.292±0.572	10.778±0.750	11.304±0.632
0.2	9.597±0.689	**11.535±0.738***	11.223±0.789	12.135±0.829	10.473±0.861
2	9.261±0.645	**14.266±0.672**‡	10.994±0.858	10.501±0.726	9.653±0.897
20	9.380±0.734	**14.840±0.539**‡	**14.921±0.581**‡	**16.403±0.562**‡	12.028±0.896
200	10.061±0.694	**15.165±0.527**‡	**15.787±0.664**‡	**16.833±0.590**‡	11.150±0.492
Estriol					
Control	9.77±0.803	8.859±0.587	9.556±0.600	10.742±0.426	10.450±0.760
0.02	9.615±0.831	**13.706±0.915**‡	**14.418±0.431**‡	10.423±1.302	10.416±0.998
0.2	9.841±0.850	**13.995±0.609**‡	**14.105±0.822**‡	11.521±1.115	11.128±0.994
2	9.389±1.063	**14.880±0.609**‡	9.640±0.825	10.623±0.834	10.866±0.955
20	9.495±0.857	9.033±0.620	9.088±0.435	9.897±0.718	10.269±0.652
200	9.934±0.862	8.955±0.599	10.183±0.507	10.581±0.750	11.246±0.591
17A-ethynylestradiol					
Control	12.675±0.714	12.232±0.562	12.567±0.500	12.994±0.459	11.954±0.600
0.02	12.830±0.548	13.478±0.507	12.139±1.143	12.835±0.616	11.835±0.622
0.2	12.530±1.300	12.229±0.310	13.157±0.910	13.188±0.644	12.484±0.768
2	11.770±0.738	12.400±0.515	**15.988±0.510***	13.735±0.664	12.668±0.516
20	11.444±0.979	**16.514±0.516**‡	**17.907±0.578**‡	**18.635±0.957**‡	**16.243±0.600**‡
200	11.095±0.854	**17.310±0.373**‡	**17.458±0.428**‡	**18.439±0.580**‡	**17.412±0.388**‡

Percentage of positive sperm head tyrosine phosphorylation. Mean±S.E.M. Differences were analyzed by KW-ANOVA; *post-hoc* comparison was performed by multiple comparisons of mean ranks. *$P<0.05$, and ‡$P<0.001$, comparison with control.



which were capacitated for 30 min and then further incubated for the standard 60 min with CaI (Supplementary Figure 2B and C). The same effect as in the case of natural estrogens was also observed for 17A-ethynylestradiol for the highest concentration of 200 ng/ml after 30 min sperm capacitation with a further incubation with CaI for 5 min (Fig. 3D). Moreover, statistically significant differences between 17A-ethynylestradiol compared with the control, as well as to other natural estrogens, were persisting for 2, 20, and 200 ng/ml over 30 and 60 min capacitation with a further incubation with CaI for the standard 60 min (Supplementary Figure 2B and C). The comparison between estrogens during CaI-induced acrosome reaction is shown for the concentration of 2 ng/ml (Supplementary Figure 2D).

Estrogens increase overall sperm protein TyrP during capacitation in vitro

As shown in Fig. 4, natural estrogens increase the number of protein bands phosphorylated on tyrosine residues as well as the staining of certain bands. Compared with the control, all concentrations of E_2, estrone, and E_3 increased TyrP. 17A-ethynylestradiol, increased TyrP at the three highest concentrations (2, 20, and 200 ng/ml). The increase of TyrP occurred mainly in the key times of capacitation between 60 and 90 min.

The SDS–PAGE results from the whole sperm lysate obtained during set time points of capacitation *in vitro* show an increasing effect, dependent on the time of capacitation and estrogen concentration. A higher concentration of estrogens in the capacitating medium and a greater number of proteins phosphorylated on tyrosine residues were detected. If comparing the differences between the amounts of protein TyrP changed in response to selected concentrations of estrogens during capacitation *in vitro*, 200 ng/ml of all selected estrogens led to an increased TyrP in the sperm in capacitation times of 30, 60, and 90 min. For estrone, E_3, and 17A-ethynylestradiol, the increasing level of

Figure 3 Effect of estrogens on mouse sperm tyrosine phosphorylation and acrosome reaction. (A) Linear time kinetics of a range of 17B-estradiol (E_2) concentrations on positive sperm head TyrP. (B) Log concentration kinetics of 17B-estradiol at various time of capacitation. (C) Log concentration kinetics of the four studied estrogens at 30 min of capacitation. (D) Log concentration kinetics of the four studied estrogens at 30 min of capacitation and 5 min CaI-induced acrosome reaction. (E) Linear time kinetics of a range of 17B-estradiol concentrations on acrosome reaction up to 90 min. For all panels, data represent an average of five replicates as described in 'Materials and Methods' section. Mean ± S.E.M. are show in Tables 1 and 2 due to a clear transparency of all graphs. The arithmetic mean of total S.E.M. was 0.970 and the S.D. of the mean of S.E.M. was 0.553. In logarithmic graphs, the plotted value for 0.0001 ng/ml represents the zero control.

Table 2 Effect of estrogens on mouse sperm acrosome reaction.

	Time of capacitation + calcium ionophore stimulation (min)					
Concentration (ng/ml)	cap0 bez CaI	cap30+CaI5	cap30+CaI60	cap60+CaI60	cap90+CaI60	cap120+CaI60
17B-estradiol						
Control	3.490±0.323	15.248±0.717	47.719±1.056	61.721±1.281	69.340±0.936	77.613±0.997
0.02	3.333±0.460	14.069±0.639	48.274±2.221	61.212±1.295	69.956±1.354	78.501±1.704
0.2	3.663±0.484	**10.035±0.471**‡	47.984±1.718	59.661±1.758	70.491±2.048	79.924±2.155
2	3.579±0.263	**9.621±0.532**‡	48.447±1.433	62.147±1.760	71.345±1.238	77.148±1.390
20	3.200±0.329	**8.873±0.411**‡	49.111±1.864	58.969±1.553	70.587±0.824	78.454±1.486
200	3.545±0.435	**8.122±0.707**‡	47.639±2.238	62.310±1.687	71.312±1.566	77.323±1.559
Estrone						
Control	2.706±0.352	10.496±0.556	37.639±0.931	57.166±1.700	71.955±1.636	82.069±1.015
0.02	2.783±0.262	8.631±0.744	39.005±1.180	56.876±2.043	72.662±2.405	81.118±2.051
0.2	2.413±0.216	9.907±0.366	38.361±1.229	57.891±1.201	71.505±0.961	82.645±1.509
2	2.409±0.316	**6.839±0.281**‡	38.567±1.783	56.962±2.039	70.677±1.064	82.245±1.966
20	2.129±0.312	**6.123±0.644**‡	37.707±1.051	54.460±2.418	69.302±2.105	80.622±1.604
200	2.433±0.272	**5.524±0.297**‡	38.780±1.700	54.528±1.973	70.377±1.601	80.002±1.777
Estriol						
Control	2.420±0.218	9.307±0.645	35.306±1.209	50.054±1.205	65.100±1.978	76.940±1.144
0.02	2.403±0.270	**6.129±0.341***	33.733±1.190	49.755±1.726	60.868±2.032	72.425±1.729
0.2	2.330±0.197	**5.598±0.669**†	**27.559±1.226**†	48.668±0.951	58.127±1.640	74.397±2.662
2	2.435±0.279	**5.462±0.500**†	35.705±1.539	48.380±2.306	57.403±2.566	78.032±1.687
20	2.589±0.248	9.198±0.728	35.779±1.298	51.440±2.533	56.193±2.075	75.098±1.829
200	2.392±0.339	8.238±0.826	34.529±1.360	50.193±2.590	55.995±1.968	75.738±1.870
17A-ethynylestradiol						
Control	4.665±0.573	12.417±0.996	33.266±0.993	42.766±1.093	62.496±1.293	75.341±1.398
0.02	4.611±0.844	11.586±0.771	31.969±1.059	43.093±1.193	61.583±2.205	73.590±2.054
0.2	4.738±0.513	10.446±0.575	33.145±1.133	40.015±1.246	61.435±1.283	75.087±2.033
2	4.555±0.641	10.641±0.330	**24.550±0.870**‡	**33.875±0.866**‡	62.834±1.098	73.414±1.779
20	4.580±0.493	11.242±1.022	**23.194±0.928**‡	**35.270±1.211**†	60.243±2.086	73.410±1.254
200	4.379±0.887	**7.157±0.734**†	**22.889±0.914**‡	**33.389±1.632**‡	63.528±2.378	76.496±1.708

Percentage of sperm after completed acrosome reaction. Mean±S.E.M. Differences were analyzed by KW-ANOVA; *post-hoc* comparison was performed by multiple comparisons of mean ranks. *$P<0.05$, †$P<0.01$, and ‡$P<0.001$, comparison with control.

TyrP was persisting up to 120 min of capacitation. The 20 ng/ml concentration elevated the protein TyrP in both estrone and E_3 at 30, 60, 90, and 120 min of capacitation. Unlike estrone and E_3, 20 ng/ml E_2 and 17A-ethynylestradiol triggered an increase of TyrP in 60 and 90 min. The concentration of 2 ng/ml gave similar results for all estrogens when E_2 and estrone TyrP were elevated in 30, 60, and 90 min for E_3 in 30 and 60 min and for 17A-ethynylestradiol in 60 and 90 min. Very similar results were obtained for the 0.2 ng/ml concentration of estrogens, where E_2, estrone, and E_3 significantly increased TyrP at 30 and 60 min and estrone at 60 and 90 min of capacitation, when comparing the results of the lowest 0.02 ng/ml concentration of estrogens, E_2, estrone, and E_3 elevated TyrP at 60 min and E_3 at 30 and 60 min of capacitation.

The results show a very similar response to selected concentrations of all four estrogens in terms of the detection of protein TyrP from the whole sperm lysate at experimental capacitation times. The general pattern of estrogen response resulting in elevating TyrP can differ slightly between capacitation times but usually the final outcome of elevated TyrP is overlapping at standard key times of mouse capacitation, which are between 60 and 90 min.

Differences between control samples and samples of sperm affected by different concentrations of estrogens were detected by gel densitometry (see 'Materials and Methods' section).

Comparison between spontaneous and CaI-induced acrosome reaction in control

The proportion of spontaneous and CaI-induced acrosome reaction in control sperm samples at all experimental times during sperm capacitation *in vitro* in shown in Fig. 5. There was a significantly increased number of acrosome-reacted sperm in the CaI-induced group (Mann–Whitney *U* test, **$P=0.0043$/35 min, ***$P<0.001$/90 and 120 min) compared with a group with spontaneous acrosome reaction rate for all experimental times except the time zero representing less than a minute with or without an induction of acrosome reaction.

Discussion

Estrogens were considered only as female hormones, but they play an important role in the male reproductive system too. Testosterone and androstenedione are

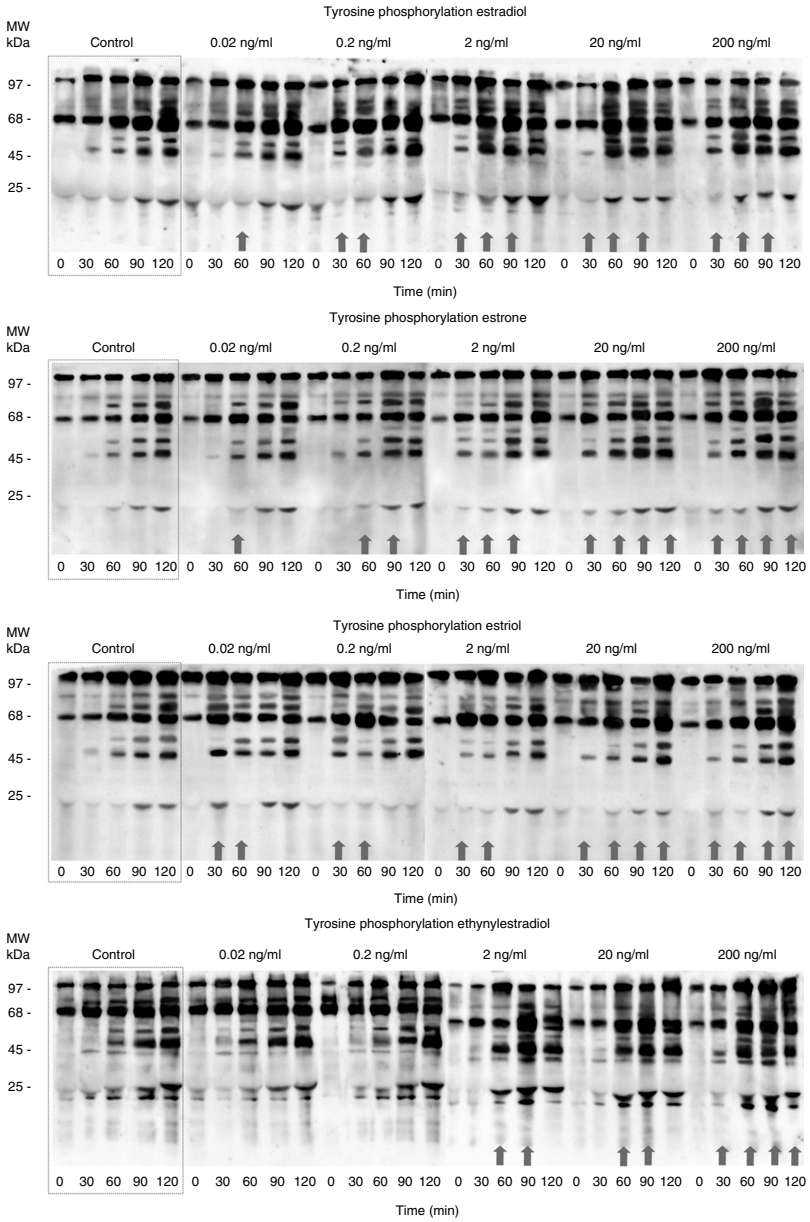


Figure 4 Increasing effect of estrogens on protein TyrP during capacitation *in vitro*. Estrogen concentrations 0.02, 0.2, 2, 20, and 200 ng/ml. Time of capacitation 0, 30, 60, 90, and 120 min. Every sample contained a protein equivalent of 10^6 cells. Representative results shown.

precursors for the synthesis of E_2 and estrone by cytochrome P450 aromatase. Consequent changes take place in the liver, where estrone is changed to E_3. This implies that estrogens are also produced in males and not only in females; therefore, they influence male reproductive parameters (Broeder *et al*. 2000). An estrogen action and specific cellular response are triggered through binding of these hormones to complementary ERs. ERs play an important role in activating signaling pathways leading to sperm capacitation, essential for further successful fertilization. ERs have been detected in the sperm of many species, for example, human (Aquila *et al*. 2004, Solakidi *et al*. 2005), boar (Mutembei *et al*. 2005), rooster (Kwon *et al*. 1995), rat (Pellettier *et al*. 2000), but failed to be detected so far in mouse spermatozoa. In this study, the 64 kDa protein corresponding to ERB was detected to be present in sperm from cauda epididymis using immunoprotein detection. Moreover, localization of ERB was defined by immunofluorescent labeling as a thin sickle localized over the apical region of sperm head, covering also the apical hook region. While the presence of ERB on mature mouse epididymal sperm was confirmed, it could be considered to take part in activating signaling pathways leading to TyrP, during capacitation. GPR30 receptor could be another receptor responsible for interaction with estrogen hormones in sperm (Aquila *et al*. 2004); however, there is a recent work questioning its E_2-mediating role in mammalian reproductive organs (Otto *et al*. 2009).

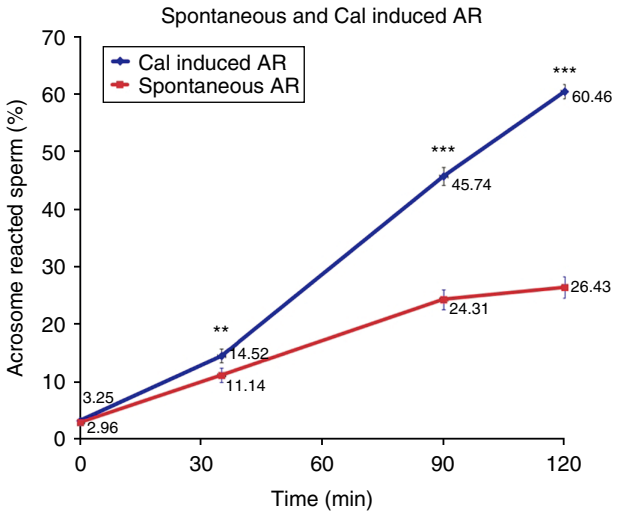


Figure 5 The comparison of spontaneous and CaI-induced acrosome reaction in control samples. Each data point represents five separate experimental observations. The statistical differences between a number of sperm after spontaneous and CaI-induced acrosome reaction at experimental times were analyzed by Mann–Whitney *U* test: 35 (**$P=0.0043$), 90, and 120 min (***$P<0.001$).

Protein TyrP that occurs during mammalian sperm capacitation is crucial for sperm to obtain the ability to undergo acrosome reaction. These events are, therefore, indicators of the sperm reproductive fitness leading to successful fertilization of the ova. TyrP is regulated by different intracellular pathways (Visconti *et al.* 2002) and these pathways are triggered by ligand-activated steroid and non-steroid receptors. Recently, the effect of estrogenic compounds on the reproductive potential of sperm has been studied (Breitbart & Etkovitz 2010, Carreau & Hess 2010). This paper addresses the question, whether a rising concentration of estrogens such as E_2, estrone, E_3, and 17A-ethynylestradiol influences mouse sperm capacitation and acrosome reaction *in* vitro. Based on the presented results it can be concluded that these studied estrogens significantly stimulate the capacitation progress in a concentration-dependent manner. The number of sperm capable of undergoing head TyrP, as well as the overall TyrP, was generally higher compared with the control. Except E_3, all other estrogens increased the TyrP mainly at higher experimental concentrations. However, interestingly with E_3, this effect was observed mainly in the two lowest concentrations after 30 min of capacitation and it could be interpreted as a so-called U-shape effect. The dose–response relationships of certain hormones are often nonlinear and are characterized by the dose–response relationship displaying low-dose stimulation and high-dose inhibition (Calabrese & Baldwin 2002).

Synthetic estrogen 17A-ethynylestradiol affected TyrP in the sperm head in all experimental times and its effect is different from other estrogens. Its influence on TyrP does not weaken during the capacitation period and the effect is displayed even after 120 min. 17A-ethynylestradiol compared to natural estrogens shows great stability, thanks to three strong bonds in its molecule, which make it extremely stable, and worsen its degradation in liver.

The percentage of sperm with positive head TyrP in our control samples was 8–12% in contrast to experimental groups where TyrP increased up to 18%. These results correlate with recent studies and denote that during sperm capacitation the percentage of sperm head TyrP in control is ~9% compared to 3% TyrP in sperm heads when incubated for 90 min in non-capacitating medium (Asquith *et al.* 2004). However, one could expect that in the beginning of the sperm capacitation the amount of sperm positive for sperm head TyrP would be lower than that in a fully capacitated population. This was not, however, so obvious in our study. This could be explained by the fact that even at the starting time a sperm suspension was exposed to a complete capacitating medium for almost a minute; therefore, sperm were still subjected to all relevant ions and proteins. For this reason, we cannot rule out the possibility that signaling pathways could have been activated. Also, in our study, the sperm from a very distal region of cauda epididymis were used in contrast to study of Asquith *et al.* (2004) who used sperm from the whole cauda epididymis.

It needs to be stated that TyrP like capacitation does not take place synchronously (Stewart-Savage 1993); however, only those fewer than 15% of free-swimming population of sperm that are tyrosine phosphorylated can recognize zona pellucida.

The presented results show that estrogens in general increase sperm TyrP, however, each estrogen can trigger a response of different strength with respect to its concentration and also capacitation time. A possible explanation of a non-identical estrogenic response may be due to the fact that estrogens activate diverse types or parts of signaling pathways, or bind to and activate different receptors triggering pathways leading to sperm TyrP during capacitation.

Simultaneously the effect of estrogens on the number of acrosome-reacted sperm after CaI-induced acrosome reaction was studied. CaI is usually used for the activation of releasing lytic proteins from the acrosome and simulates the *in vivo* zona pellucida triggered acrosome reaction (Yamagata *et al.* 1998). All studied estrogens affected the onset of acrosome reaction; however, in this case, estrogens significantly reduced the percentage of sperm that completed the acrosome reaction. These results correlate with recent studies (Baldi *et al.* 2000, Vigil *et al.* 2008), which show a decreased ability of sperm to undergo the acrosome reaction after their capacitation with E_2. Similarly, our results summarizing the state of the acrosome after CaI-induced acrosome reaction correlate with an elevated amount of sperm head TyrP during sperm capacitation in presence of estrogens. On the other hand, it was presented (Adeoya-Osiguwa *et al.* 2003) that E_2

stimulated acrosome reaction in uncapacitated sperm, but in capacitated sperm it had no effect. These results, however, are not in contrary to ours, as the acrosome reaction was not initiated in uncapacitated sperm, and moreover, no changes between the experimental groups and controls were observed after 90 min of capacitation, when this is more or less complete. The observed decrease of sperm ability to undergo acrosome reaction was obtained for sperm after 30 min of capacitation. On the other hand, those sperm capacitated with natural estrogens for a longer period did not show differences to the control, suggesting either the slowing down of capacitation or an effect that is lost or compensated in fully capacitated sperm. This was not the case, however, with 17A-ethynylestradiol, where a decreased acrosome reaction persisted in sperm capacitated for 60 min, when mouse capacitation is completed. This proposes its prolonged adverse effect on the sperm-fertilizing ability.

In mice, there is a high spontaneous acrosome reaction (Johnson *et al*. 2007), whose rate depends on time of capacitation. The proportion of spontaneous and CaI-induced acrosome reaction gives, therefore, important information to assess the magnitude of the actual effect of estrogens on CaI-induced acrosome reaction. The decrease of sperm ability to undergo the induced acrosome reaction in presence of selected estrogens falls in early capacitating times below the spontaneous acrosome rate, which may emphasize the actual effect of estrogens on ability of sperm to undergo the acrosome reaction.

The results of TyrP from immunofluorescent analysis were confirmed by immunoprotein detection of the whole sperm samples. The characteristics of specific changes in the sperm head are crucial for judging the sperm ability to fertilize (Stewart-Savage 1993). For this reason, we have predominantly focused on the effect of different concentrations of selected estrogens on the TyrP in the sperm head. On the other hand, the status of the whole sperm considering TyrP in the flagellum could not be ignored. The SDS–PAGE results show that selected estrogens increase the overall protein TyrP in the whole sperm lysate during capacitation *in vitro*. In correlation with previously published results (Visconti *et al*. 1995*a*), our control protein samples showed a time-dependent increase in TyrP during mouse sperm capacitation. The results of protein phosphorylation from sperm capacitated with selected estrogens showed a significant increase in the number of proteins phosphorylated on tyrosine residues in higher concentrations for E_2, estrone, and 17A-ethynylestradiol, and in a lower concentration for E_3. As protein TyrP is ongoing in the sperm head and the flagellum, results from immunoprotein detection of the whole sperm lysate samples and the immunofluorescence detection of TyrP in the sperm head cannot be fully compared. Nevertheless, some correlation could be made and a parallelism can be seen between these two groups of results.

Comparing currently obtained results, it is clear that each of the selected estrogen significantly affects the two 'markers' of sperm capacitation such as TyrP and a consequent acrosome reaction in the opposite way. Before sperm–egg fusion, sperm undergoes molecular changes, which are indicated by the status of TyrP (Visconti *et al*. 1999), activation of acrosome response, and sperm binding to zona pellucida (Baldi *et al*. 2002). These processes strictly follow one another in a precisely set order. Among many, TyrP triggers actin polymerization, which prevents the premature fusion of the plasma and outer acrosomal membranes leading to acrosome reaction (Brener *et al*. 2003). If the ERs are hyperstimulated by an excessive amount of estrogens, the activity of the protein tyrosine kinase is elevated and the phosphorylation on tyrosine residues remains triggered. Therefore, the phospholipase D is constantly activated leading to a consequent polymerization of actin (Breitbart *et al*. 2005). During the acrosome reaction, the activation of phospholipase C leads to an increase of intracellular Ca^{2+} ions causing actin depolymerization. However, if in the beginning of the acrosome reaction the phospholipase D remains constantly upregulated by protein tyrosine kinase, the activity of protein kinase C can be possibly delayed and depolymerization of actin slowed down. The timing of all the processes involved in the molecular changes leading to capacitation and acrosome reaction is very important and inaccurate time setting of consecutive events can result in a reduction in the sperm-fertilizing ability.

Spermatozoa face on their journey through the female reproductive tract under different concentrations of estrogen, similar to those selected in our *in vitro* experiments, depending on the phase of the cycle. Estrogens are low during the preovulatory stage and E_2 is associated with sperm longevity (Mbizvo *et al*. 1990), but the situation changes at the time of ovulation, when the concentration of estrogens released with follicular fluid rises (Shaikh 1971). In correlation to our result, the capacitation and acrosome reaction are modulated by higher concentrations of estrogens and these can serve as a sperm-specific selection barrier where inhibiting effect of estrogens on induced acrosome reaction may have a physiological relevance (Vigil *et al*. 2008). Therefore, estrogens may be seen as one of many preferred cryptic female mechanisms to select the best possible sperm to fulfil the task.

In conclusion, this study shows an existence of ERB in mature mouse spermatozoa. It also provides evidence that estrogens significantly stimulate the capacitation progress in a concentration-dependent manner. On the other hand, estrogens decrease the number of acrosome-reacted mouse sperm after the induced acrosome reaction. Based on our results, it can be inferred that a raising concentration of estrogens in the environment may represent a potential risk in altering certain mechanisms contributing to the fitness of sperm fertilization.

Materials and Methods

Animals

Inbred BALB/c mice were obtained from a breeding colony of the Laboratory of Reproduction, Faculty of Science, Charles University in Prague or purchased directly from Velaz (Unetice, Czech Republic). Mice were housed in the animal facilities and food and water were supplied *ad libitum*. Male mice used for all experiments, were in a reproductive age of 10–12 weeks. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and subjected to review by the Local Ethics Committee.

Capacitation

Sperm from the distal regions of cauda epididymis were released into M2 fertilizing medium (Sigma–Aldrich) under paraffin oil in 37 °C in 5% CO_2. Released sperm were assessed for motility and viability. Sperm stock was diluted to the required concentration (5×10^6/ml) into M2 medium under paraffin oil with five different concentrations of selected estrogens (0.02, 0.2, 2, 20, and 200 ng/ml). Sperm capacitated in M2 medium without added estrogens were used as a control. Sperm samples were collected at 0, 30, 60, 90, and 120 min of capacitation *in vitro*. The time marked as 0 was the minimum time required for sperm being added to capacitated medium, removed out of the medium, and washed. This manipulation did not exceed 1 min. These samples served as a negative control. Sperm motility and viability were assessed at every experimental time point.

Immunofluorescence detection of ERB

The distal region of mouse cauda epididymis was placed in 37 °C PBS in 5% CO_2, for 10 min. Released sperm were washed in PBS, smeared onto glass slides, air-dried, and fixed with methanol for 7 min at −20 °C followed by 10 min in 0.2% Triton X-100. For immunofluorescent labeling, sperm were blocked with 3% BSA in PBS for 1 h, followed by incubation with the primary antibody BERB H150 (sc-8974, Santa Cruz Biotechnology, Heidelburg, Germany; 1:50) in PBS at 4 °C, followed by a secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L; A11008, Molecular Probes, Grand Island, NY, USA; 1:1000) in PBS. Slides were mounted into a Vectashield Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Samples were examined with an Olympus IX81 fluorescent microscope and photographed with a Hamamatsu ORCA C4742-80-12AG, using Olympus Soft Imaging Solutions Software.

Immunofluorescent detection of TyrP

Sperm smears were air dried and fixed with 3.7% formaldehyde in PBS pH 7.34 at room temperature for 10 min, followed by washing in PBS, incubation with ammonium chloride (NH_4Cl) 15 mM for 5 min, and with 0.1% detergent Triton X-100 for 3 min. Slides were washed with PBS and left in PBS with sodium azide (NaN_3) at 4 °C. For immunofluorescent labeling, slides were blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine (PTyr 01 112630025, Exbio Antibodies, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS for 2 h, followed with Alexa Fluor 488, donkey anti-mouse IgG (A21202, Molecular Probes) secondary antibody 1:1000 in PBS for 1 h. Irrelevant isotype-matched MABs and secondary antibodies (used without primary antibodies) served as negative controls. The MAB MM10 anti-mouse CD46 (HM1118, HyCult, Uden, The Netherlands), which recognizes an acrosome-associated antigen, was used as a positive control. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Slides were examined with an epifluorescent microscope (as stated above). For every experiment we collected sperm data from 10 mice. The positive or negative signal was evaluated from a total of 200 sperm on every slide. In each group, at least five samples were analyzed. Data were analyzed statistically by Kruskal-Wallis ANOVA (KW-ANOVA); *post-hoc* comparison was performed by multiple comparisons of mean ranks; for details see 'Statistical analysis' section.

Acrosome reaction

Spermatozoa from cauda epididymis were capacitated as described earlier. During capacitation the acrosome reaction was induced by CaI (A23187 (CaI), Sigma–Aldrich) at a final concentration of 5 μM. At each experimental capacitating time of 30, 60, 90, and 120 min, the CaI was added for the standard 60 min incubation. CaI was also added into parallel sperm droplets after 30 min capacitation but only for 5 min. Sperm that served as zero control and their presence in capacitating medium did not exceed 1 min were not further induced with CaI. The control samples were also evaluated for a rate of spontaneous acrosome reaction at relevant times to CaI-induced AR at 35, 90, and 120 min. All the sperm samples were incubated at 37 °C under 5% CO_2. A drop of spermatozoa was placed onto a glass slide and 2.5 μM PNA lectin (peanut aglutinin lectin conjugate) (Molecular Probes) was added. The status of the acrosome was examined immediately under a fluorescent microscope. For every experiment we collect sperm data from 10 mice. A total of 200 cells were evaluated in each group and at least five samples were analyzed. Data were analyzed statistically by KW-ANOVA; *post-hoc* comparison was performed by multiple comparisons of mean ranks; for details see 'Statistical analysis' section.

SDS–PAGE immunoblotting

SDS electrophoresis and immunoblotting technique were used for the TyrP assessment and ERB detection. They were performed by protocols based on standard methods (Laemmli 1970, Towbin *et al*. 1979). Sperm were collected at 0, 30, 60, 90, and 120 min of capacitation *in vitro*. A suspension of noncapacitated sperm from a sperm stock released from cauda epididymis was used. The sperm solution was diluted with PBS and the number of sperm cells was counted using a Bürker chamber to ascertain a final concentration of 10^6 cells in the sample. Sperm pellet was resuspended in an equal volume of SDS–PAGE nonreduced sample buffer and heated at 97 °C for 3 min. Samples containing a protein equivalent of 10^6

capacitated sperm cells were run on a 5% stacking and 10% running SDS–polyacrylamide gel using Precision Plus Protein All Blue Standards (Bio-Rad) as MW markers. Proteins were then transferred onto a nitrocellulose membrane. Non-specific sites on the membrane were blocked with PBS-blocking solution (5% skim milk and 0.05% Tween 20). ERB was identified by primary polyclonal antibody (H150, Santa Cruz Biotechnology) against N-terminal A/B domain, diluted 1:200 and secondary goat anti-rabbit antibody conjugated to HRP (170 5046, Bio-Rad) diluted 1:20 000. Proteins phosphorylated on tyrosine residues were identified by the primary MAB anti-phosphotyrosine (PTyr 01 112630025, Exbio Antibodies) diluted 1:500 followed by a peroxidase goat anti-mouse IgG secondary antibody (A 0168, Sigma–Aldrich) diluted 1:20 000. Protein staining was visualized by chemiluminescence (Super Signal West Dura Extended Duration Substrate, Thermo Fisher Scientific, Pardubice, Czech Republic). These experiments were performed at least three times with similar results. Representative results are shown.

Gel densitometry was performed with an Aida image analyzer 4.18 (Raytest GmbH, Straubenhardt, Germany) and the relative intensity of individual signals was determined.

Statistical analysis

Experimental data were analyzed using a program STATISTICA 6.0 (StatSoft CR s.r.o, Czech Republic). The statistical differences among compared groups (number of cells with specific sperm head status in experimental samples vs control sample in appropriate time) were analyzed by one-way analysis of covariance (KW-ANOVA). The number of cells with specific sperm head status at the beginning of the capacitation process was used as a covariate to reduce the effect of differences in the capacitation status of the sperm among individual animals, which is not the result of estrogen treatment. *Post-hoc* comparison was done by multiple comparisons of mean ranks. The $*P$ value <0.05 ($**P<0.01$ and $***P=0.001$ respectively) was considered significant. The results of statistical analysis including mean$\pm$S.E.M. are presented in Tables 1 and 2.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-11-0326.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPLEMENT 3

Ded, L, N Sebkova, M Cerna, F Elzeinova, P Dostalova, J Peknicova, and K Dvorakova-Hortova 2013 In vivo exposure to 17 beta-estradiol triggers premature sperm capacitation in cauda epididymis. *Reproduction* **145** 255-263.

My contribution to this work: I generated the experimental data using CTC method and qRT-PCR, analyzed testicular sperm suspensions, performed statistical analysis of the data, contributed to the interpretation of all results and wrote the manuscript.

REPRODUCTION
RESEARCH

In vivo exposure to 17β-estradiol triggers premature sperm capacitation in cauda epididymis

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Abstract

Estrogens play a crucial role in spermatogenesis and estrogen receptor α knock-out male mice are infertile. It has been demonstrated that estrogens significantly increase the speed of capacitation *in vitro*; however this may lead to the reduction of reproductive potential due to the decreased ability of these sperm to undergo the acrosome reaction. To date the *in vivo* effect of estrogens on the ability of sperm to capacitate has not been investigated. Therefore, in this study, we exposed mice ($n=24$) to 17β-estradiol (E_2) at the concentration of 20 ng/ml either during puberty from the fourth to seventh week of age ($n=8$), or continuously from birth for a period of 12 weeks ($n=8$) at which age the animals from both groups were killed. The capacitation status of epididymal and testicular sperm was analysed by tyrosine phosphorylation (TyrP) antibody (immunofluorescence and western blot) and chlortetracycline (CTC) assay. According to our results, *in vivo* exposure to increased E_2 concentrations caused premature sperm capacitation in the epididymis. The effect of E_2, however, seems reversible because after the termination of the exposure premature epididymal sperm capacitation is decreased in animals treated during puberty. Furthermore the changes in epididymal sperm capacitation status detected by TyrP and CTC positively correlate with plasma levels of E_2 and the expression of the estrogen-dependent trefoil factor 1 (*Tff1*) gene in testicular tissue. Therefore, our data implicate that *in vivo* exposure to E_2 under specific conditions leads to the premature capacitation of mouse sperm in epididymis with a potential negative impact on the sperm reproductive fitness in the female reproductive tract.
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Introduction

Estrogens play a key role during male reproduction including spermatogenesis (Carreau *et al*. 2011, 2012) and sperm maturation such as capacitation (Baldi *et al*. 2009). Estrogen response is mediated through both genomic and non-genomic actions, where the genomic one involves binding to estrogen receptors ERs and nuclear transcription factors that activate the expression of target genes. The non-genomic estrogen action happens through transmembrane receptors such as ERA, ERB and G-protein coupled receptors (GPR30), triggering rapid intracellular signaling pathways, including the activation of serine/threonine and tyrosine phosphorylation (TyrP), leading to sperm capacitation (Kalab *et al*. 1998, Filardo *et al*. 2002). As a consequence of phosphorylation on mainly tyrosine residues, which is the key marker of successfully ongoing capacitation (Visconti *et al*. 1995*a*,*b*), the cytoskeleton protein network changes its dynamics, and acrosome reaction (AR) can occur (Yanagimachi 1994). Interestingly, progesterone-induced AR is inhibited by the extra amount of 17β-estradiol (E_2) through interaction with a specific non-genomic estrogen receptor on the sperm plasma membrane, suggesting that E_2, present at micromolar levels in follicular fluid, may act as a physiological modulator of sperm progesterone response, ensuring the appropriate timing of activation in the fertilisation process (Luconi *et al*. 1999). In rat and mouse males, the concentration of E_2 in blood plasma is 2–25 pg/ml, and it is lower than that in rete testis fluid (250 pg/ml; Free & Jaffe 1979). On the other hand, the concentration of estrogen in ovarian fluid is much higher when compared with the plasma (Free & Jaffe 1979, Hess *et al*. 1995) and it reaches multiple values depending on the time of the estrus fluctuating between 145 and 2100 pg/ml in rats and mice (Shaikh 1971). Most of our knowledge regarding these processes has been obtained from *in vitro* studies (Adeoya-Osiguwa *et al*. 2003, Ded *et al*. 2010, Sebkova *et al*. 2012), and for this reason it is really important to also do *in vivo* experiments.

Recent studies have shown that exposure to environmental estrogens may also influence male fertilising

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capability (Akingbemi 2005, Mathur & D'Cruz 2011). This includes chemicals that occur naturally in plants such as genistein and resveratrol (phytoestrogens) and also man-made chemicals such as bisphenol-A, diethylstilbestrol and vinclozolin (xenoestrogens), which can disrupt the endocrine function of animals and negatively influence spermatogenesis and sperm parameters (Peknicová *et al*. 2002, Kyselova *et al*. 2003, 2004, Elzeinova *et al*. 2008). These chemicals again mainly act by binding to estrogen receptors (Kuiper *et al*. 1998, Sohoni & Sumpter 1998, Akingbemi & Hardy 2001) and trigger a rapid non-genomic signaling response. The key estrogen receptor is ERA as its knock-out mice are infertile due to the disruption of the estrogen action within somatic cells of the reproductive system (Dupont *et al*. 2000). ERA activity has been demonstrated by expression of the trefoil factor 1 (*Tff1*) gene (Kim *et al*. 2000, Park *et al*. 2012), which is also a marker gene for the analysis of estrogenic activity (Dorosh *et al*. 2011).

The objective of this study was to test the *in vivo* effect of E_2 on the level of TyrP as a marker of ongoing mouse sperm capacitation and to monitor the *Tff1* gene expression as the marker for ERA activity.

Results

In vivo *exposure to E_2 increases the TyrP of epididymal sperm head proteins*

The number of positively labelled sperm heads for TyrP (Fig. 1) at the start of capacitation (time 0) was significantly higher in both experimental groups with pubertal (A) and continuous exposure to E_2 (B) compared with the control (Fig. 2). The difference between the control and experimental pubertal E_2 group was on the border of significance ($P \leq 0.05$). On the other hand, the difference between the control and experimental continuous E_2 group was highly significant ($P \leq 0.001$) despite the fact that the variance in the continuous E_2 samples was higher compared with the control and pubertal E_2 samples. Moreover, the difference between pubertal E_2 and continuous E_2 was also significant ($P \leq 0.01$), thus indicating that sperm cells from animals after continual exposure to E_2 (continuous E_2) are highly different to those after pubertal exposure (pubertal E_2) and the control.

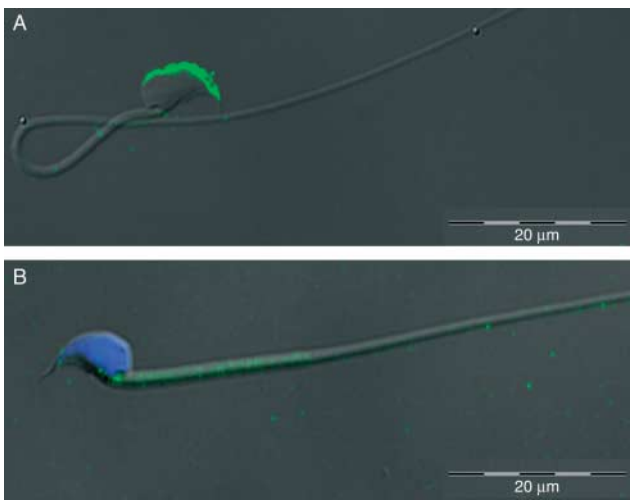

Figure 1 Immunoflourescent detection of tyrosine phosphorylation (TyrP) in mouse epididymal sperm. (A) TyrP-positive sperm head and (B) TyrP-negative sperm head. Scale bar 20 µm.

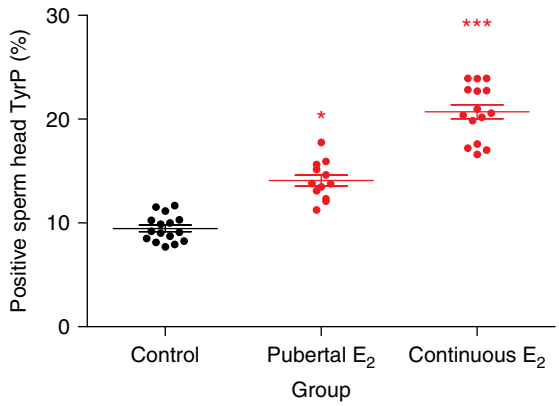

Figure 2 Number of capacitated epidymidal sperm detected by anti pY antibody (time 0). Control group, pubertal E_2 and continuous E_2. Bars denote arithmetical means of capacitated cells (%), whiskers denote S.E.M. and points denote individual measurements. *$P<0.05$, ***$P<0.001$.

In vivo *exposure to E_2 changes the distribution of chlortetracycline fluorescent patterns in epididymal sperm cells*

In addition to the analysis of the sperm head TyrP, chlortetracycline (CTC) analysis was performed to evaluate changes in CTC fluorescent patterns relating to sperm capacitation (Fig. 3). Data from the CTC analysis correlate with the data from TyrP analysis. The control group had the lowest number of cells with CTC pattern B relating to the capacitated status of the sperm (Fig. 3). Similar to the data from TyrP analysis, the number of cells with CTC pattern B (Fig. 3) relating to the capacitated status was significantly higher in both experimental groups compared with the control (Fig. 4). The difference between the control and pubertal E_2 was the lowest ($P \leq 0.05$), and the difference between the control and continuous E_2 was the highest ($P \leq 0.001$). Also the difference between the two experimental groups, pubertal E_2 and continuous E_2, was significant. This fact indicates the significant changes in the distribution of the CTC fluorescent patterns after the termination of E_2 exposure in the pubertal E_2 group. Together, with the data from TyrP labelling, western blot (WB; Fig. 5) and CTC analyses provide strong evidence for the procapacitation effect of E_2 based on the analysis of these two parameters. Also the correlation between the number of TyrP-positive sperm heads and the number of spermatozoa with the pattern B was high ($r=6.683$, $P=0.0358$).

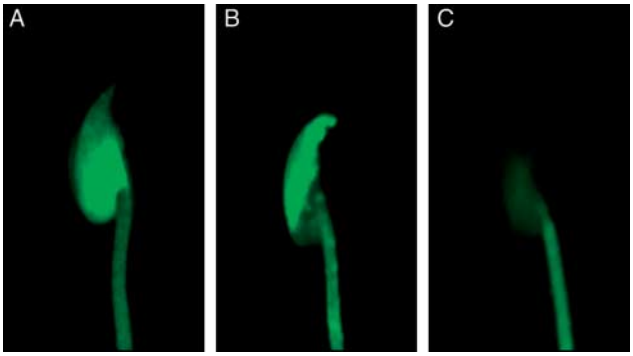

Figure 3 Patterns of fluorescence in CTC staining. (A) Bright fluorescence over the entire head with a brighter line of fluorescence across the equatorial segment (pattern F, non-capacitated spermatozoon). (B) Bright fluorescence in the anterior segment of the head with an absence of fluorescent labelling over the equatorial and post-equatorial segments (pattern B, capacitated spermatozoon). (C) Low or absent fluorescence over the entire head (pattern AR, acrosome-reacted spermatozoon).

The initial differences of the sperm head TyrP are propagated during in vitro *capacitation*

After analysis of the native epididymal sperm suspension, sperm were capacitated to assess the potential differences in the capacitation progress at individual capacitation times. At the start of the capacitation process, there exist significant differences between the control and experimental groups representing the differences in the phosphorylation of the sperm head proteins directly in the epididymis (Fig. 6). These differences were propagated into the subsequent capacitation times. After 30, 60, 90 and 120 min of capacitation, the differences between the control and experimental groups followed those from the time 'zero' representing a positive epididymal sperm head TyrP. The highest, but not statistically significant, difference between the capacitation progress was at 30 min of capacitation (time) for continuous E_2 (Fig. 7). This difference indicates the fact that some population of the spermatozoa from animals exposed to E_2 is on the border of the capacitation status and after the initial capacitation impulse, this population responds by increasing the total amount of capacitated spermatozoa in the first subsequent capacitation time (30 min; Fig. 7). The data from positive TyrP labelling were further supported by WB analysis of TyrP (Fig. 5). The difference between the control and pubertal E_2 is not visible at the time 0. On the other hand, there is a higher TyrP of proteins around 68 and 45 kDa for continuous E_2. Therefore, the evidence from WB partially supports data from TyrP fluorescence, despite the fact that WB also included differences between TyrP of the tail-associated proteins, which were not considered during the TyrP fluorescence analysis.

The indicators of capacitation status positively correlate with the serum levels of E_2 and expression of Tff1 *gene*

For the analysis of the connection between the indicators of sperm capacitation status and other relevant physiological parameters of estrogen action, the measurement of E_2 serum levels (Fig. 8A) and expression of the estrogen-dependent *Tff1* gene (Fig. 8B) were analysed and statistically correlated with the previous results from the sperm analysis. According to the obtained results, the percentage of the TyrP-positive sperm head cells in the epididymis positively correlates both with E_2 plasma levels and with the expression of the *Tff1* gene in the testicular tissue. The concentration of plasma E_2 in the control group and pubertal E_2 group was relatively similar with the arithmetical mean around 46 pg/ml and had a S.D. of 17. On the other hand, plasma E_2 levels in Continuous E_2 group were much higher and reached an average concentration of 3705 pg/ml with an S.D. of 205. The correlation coefficient r between the number of TyrP-positive sperm cells and plasma levels of E_2 was 0.549 ($P \leq 0.05$), which indicates a high correlation between these two parameters (Fig. 8A). Also the correlation between the expression of the *Tff1* gene expressed as C_q values and the number of TyrP-positive sperm were high ($r = -0.881$, $P \leq 0.001$) indicating a strong connection between these two parameters (Fig. 8B).

The TyrP of testicular epididymal sperm head proteins is not altered after in vivo *exposure to E_2*

To examine the potential effect of E_2 exposure on the capacitation status of testicular sperm, the TyrP of epididymal sperm head proteins was analysed (Fig. 9). In the control and both experimental groups, the percentage of TyrP-positive sperm heads was relatively low (about 1.5%) with no significant differences between

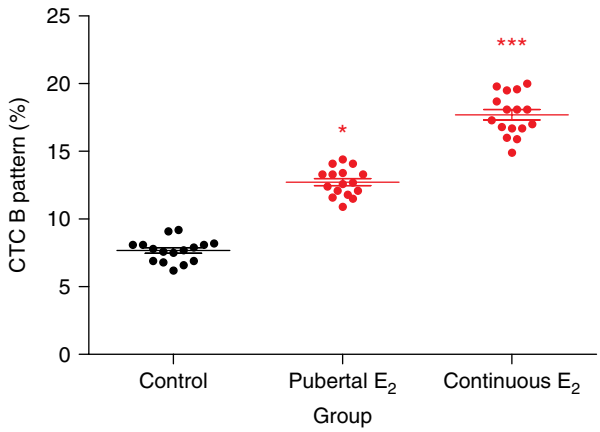

Figure 4 Number of capacitated epidymidal spermatozoa detected by CTC staining (time 0). Control group, pubertal E_2 and continuous E_2. Bars denote arithmetical means of capacitated cells (%), whiskers denote S.E.M. and points denote individual measurements. *$P<0.05$, ***$P<0.001$.

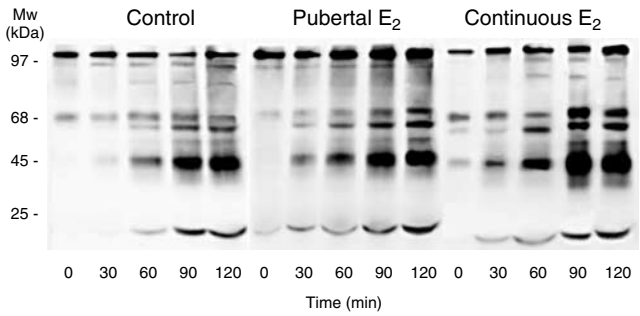

Figure 5 Western blot analysis of protein phosphorylation during *in vitro* capacitation detected by anti pY antibody. Control group (C), pubertal E_2 and continuous E_2. The higher phosphorylation of proteins at the start of capacitation (bands around 68 and 45 kDa) in continuous E_2 is visible. The highest cumulative protein phosphorylation in subsequent capacitation intervals is visible.

control and experimental groups and also without any significant difference between the two experimental groups ($P \geq 0.05$; Fig. 10). Owing to the fact that no significant differences among the groups were observed, the power analysis for the statistical test was performed and it was 0.227.

Discussion

Estrogens play an important role in almost all sperm physiological processes from the production of sperm in the testes to fertilisation in the female oviduct. For the last decade, the effect of estrogens on specific physiological processes in sperm has largely been studied during *in vitro* experiments. In these experiments, natural and synthetic estrogens have shown a procapacitation concentration-dependent effect on epididymal (mouse; Sebkova *et al.* 2012) and ejaculated spermatozoa (boar; Ded *et al.* 2010). Exposure to estrogens during *in vitro* capacitation provides a good model for simulating the *in vivo* conditions in the female reproductive tract. On the other hand, sperm are exposed to estrogens and various compounds with estrogenic activity from the time of their production to their release into the testicular tubule. Owing to the fact that some of the sperm already express specific indicators of the capacitation status in the epididymis, it is important to know whether or not the sperm capacitation status can be altered during sperm storage in the cauda epididymis in response to the estrogen stimuli. This type of study required *in vivo* exposure to estrogens; therefore, the most common and well-studied E_2 was selected for this purpose. We have shown that molecular capacitation markers in sperm after *in vivo* exposure to E_2 are similar to those observed *in vitro*. Both TyrP and CTC pattern distribution were changed in a similar way after *in vitro* and *in vivo* exposure.

Contrary to previous *in vitro* experiments, during *in vivo* exposure, E_2 does not reach sperm cells directly, but it has to pass through multiple biological barriers before it is able to have a possible effect on epididymal sperm. The normal serum concentration of E_2 is at the range of 2–20 pg/ml (Snyder *et al.* 2009). There is also evidence that in the rete testis the concentration of estrogens is tenfold higher and related to the plasma levels (Free & Jaffe 1979). In our study, the extensiveness of the E_2 effect on the sperm physiological parameters positively correlates with the serum levels. We also measured the expression of the *Tff1* gene, the specific estrogen response gene in the testicular tissue. The correlation between the expression of this gene and physiological parameters of the spermatozoa was also positive and more significant compared with the plasma levels of E_2. Therefore, the extensiveness of the molecular changes in epididymal sperm cells is largely dependent on the plasma and tissue levels of E_2. After exposure the phosphorylation status and CTC patterns relating to calcium homeostasis then returned to levels more similar to the control ones, which may show that the effect can be reversible if the exposure is terminated. Despite this process, there are still differences between the control and experimental pubertal E_2 groups in the number of cells with capacitated patterns. This situation may be related to the fact that although there are no differences in the plasma concentration of estrogens the concentration in the reproductive tract could be still higher. The higher expression of *Tff1* gene in pubertal E_2 supports this hypothesis. On the other hand, the molecular changes initiated by estrogens on the level of spermatogenesis and propagated into the subsequent developmental changes of the sperm cannot be excluded, as the gene expression pattern is altered weeks after the exposure and could be propagated by epigenetic mechanisms to subsequent generations.

During *in vivo* exposure the estrogens can also influence the future sperm capacitation ability during

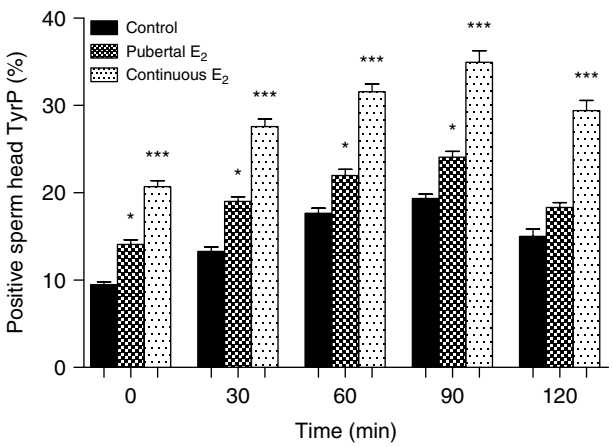

Figure 6 Number of capacitated spermatozoa after 0, 30, 60, 90 and 120 min of *in vitro* capacitation. Capacitation progress was measured by anti pY antibody (Fig. 1). $*P<0.05$, $***P<0.001$.

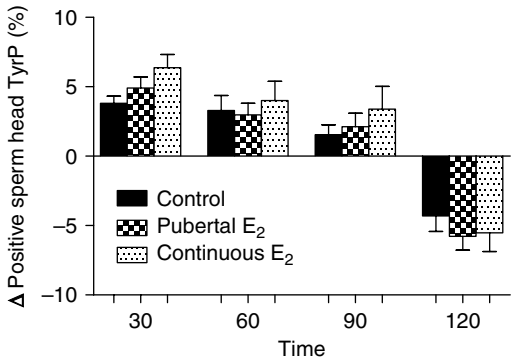

Figure 7 Differences (Δ) in the number of capacitated spermatozoa detected by anti pY antibody between two subsequent capacitation times (30–0, 60–30, 90–60, 120–90) in individual control and experimental groups.

spermatogenesis and sperm storage. Therefore, the analysis of the capacitation status of testicular sperm has been performed in the current study. During this analysis, there were no significant differences in the number of TyrP-positive testicular spermatozoa among the control and experimental groups. Based on the results of a previous *in vitro* study (Sebkova *et al.* 2012), it can be assumed that with an elevated *in vivo* estrogen exposure, capacitation may start in the epididymal spermatozoa. On the other hand, it has been reported that estrogens trigger multiple phosphorylation pathways in the testicular tissue or can change their cell sensitivity (Miyaso *et al.* 2012). For this reason it is possible to consider that potential molecular changes in testicular sperm, and individual sperm developmental stages can lead to a higher number of capacitated sperm observed in the epididymis. As shown in this study the sensitivity (statistical power) of testicular sperm head TyrP analysis was lower compared with the epididymal one and therefore the potential differences in this parameter in testicular tissue cannot be fully excluded.

The premature molecular changes related to the capacitation process may lead to many consequences after ejaculation and physiological capacitation in the female reproductive tract. The precise timing of individual molecular processes during the capacitation is an absolute prerequisite for successful fertilisation. From the population of millions of sperm there are only tens or hundreds reaching the egg and only one can successfully fertilise it. Already at the start of capacitation, sperm cells are unequal concerning their morphology and ability to reach, penetrate and fertilise the egg. It has been recently shown that higher TyrP can lead to a decreased ability of sperm to undergo AR in the presence of calcium-ionophore (Sebkova *et al.* 2012). The hyperphosphorylation of sperm proteins may therefore lead to the inability of sperm to undergo AR.

On the other hand, calcium changes detected by the CTC method are prerequisite for physiological AR and egg fertilisation. In general, considering a wide range of mammalian species, including humans, the premature calcium influx induced by estrogens may lead to premature AR without the presence of zona pellucida factors. Together, with the hyperphosphorylation of sperm proteins the percentage of spermatozoa with an ideal physiological status relating to their ability to reach and fertilise the egg strongly decreases.

The population of epididymal spermatozoa is different concerning its maturation and 'capacitation' status. This diversity seems to be a good criterion for successful fertilisation, due to the high number of spermatozoa, as it is usual in each sperm population that there is a subpopulation of spermatozoa with ideal molecular characteristics to fertilise the egg. Estrogens and other

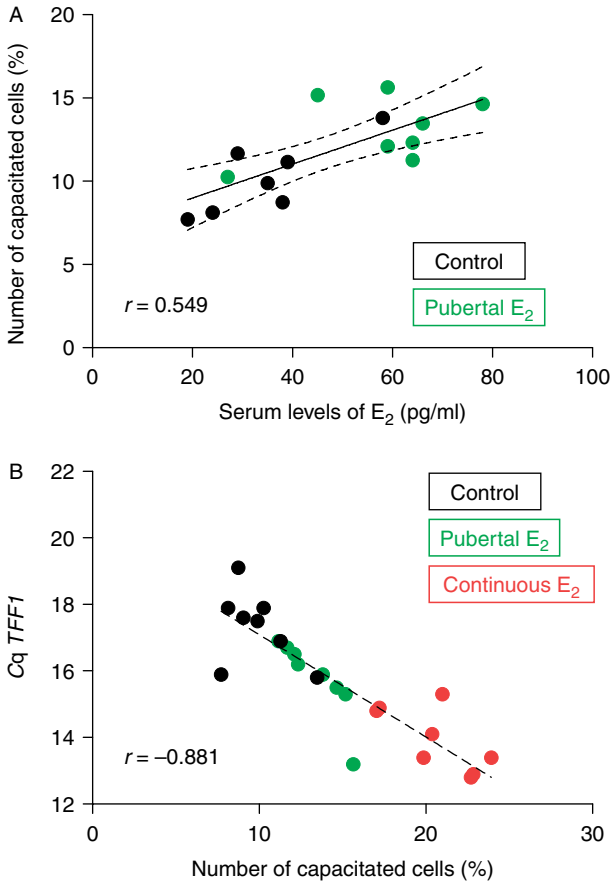

Figure 8 (A) The correlation between the number of capacitated epidymidal sperm (time 0 of the capacitation) detected by anti pY antibody and E_2 plasma levels detected by RIA in pubertal E_2 and control groups. Each data point represents the measurement of both parameters from one animal. Individual groups are labelled with different colours. The highly different continuous E_2 group was excluded from this analysis. The straight line indicates the Pearson coefficient (r), the dotted lines indicate (95%) confidence intervals. (B) The correlation between the number of capacitated epidymidal spermatozoa (time 0 of the capacitation) detected by anti pY antibody and the expression of the estrogen-dependent *Tff1* gene. The expression of the *Tff1* gene is expressed as the normalised C_q values. Each data point represents the measurement of both parameters from one animal. Individual groups are labelled with different colours. The dotted line indicates the Pearson coefficient (r).

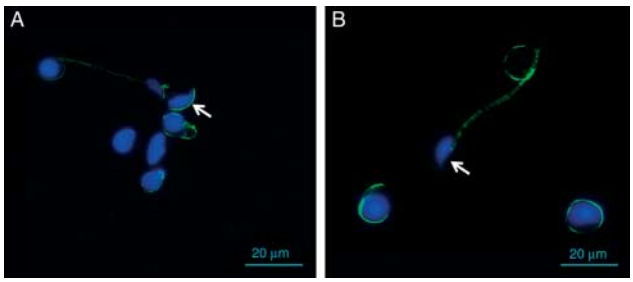

Figure 9 Immunofluorescent detection of TyrP in testicular sperm head. (A) Positive testicular sperm head (indicated by an arrow), (B) negative testicular sperm head. Scale bar 20 μm.

compounds with estrogenic activity can affect the balance of the capacitation status directly in the epididymis and through this activity they may decrease the fertilising potential of spermatozoa. After terminating the E_2 exposure, the situation more or less went back to normal, which was close to the status of the control group; therefore, this effect seems to be reversible. Based on our data, it is also clear that *in vivo* E_2 exposure influences the proportion of spermatozoa with the specific molecular characteristics relating to only the beginning of the capacitation. During the subsequent *in vitro* capacitation, the differences between the control and experimental groups remained the same as observed at the beginning. It implies that the sperm population, of the exposed animals reaching the egg could have a significantly altered capacitation status and a lower fertilising potential compared with the control ones.

The premature sperm capacitation in the cauda epididymis may be one of the factors bringing a negative effect of the endocrine-disrupting chemicals on the male reproductive functions. It has been recently shown that not only natural but also artificial estrogens significantly affect the sperm capacitation process (Sebkova *et al*. 2012). Although 17α-ethynylestradiol only seems to act at higher concentrations (≥2 ng/ml), the combination of all substances with potential or confirmed estrogenic activity can further misbalance the physiology of epididymal spermatozoa. Furthermore some artificial compounds with estrogenic activity have a significant effect on sperm physiology at lower doses compared with the strongest natural estrogen such as E_2 (Fraser *et al*. 2006, Sebkova *et al*. 2012).

In conclusion, our data imply that *in vivo* exposure to E_2 leads to premature 'capacitation' of mouse sperm in the cauda epididymis with a further potential negative impact on sperm reproductive fitness in the female reproductive tract. This effect is caused mainly by the hyperphosphorylation of sperm proteins and a/the premature calcium influx. These processes lead to a decreased ability of sperm to undergo an AR. Based on this evidence, *in vivo* exposure to E_2 can lead to a decrease of the fertilising potential in male mice with significant relevance to endocrine-disrupting compounds with estrogenic activity.

Materials and Methods

Animals and E_2 exposure protocol

Inbred mice (Anlab, Prague, Czech Republic) were housed in an animal facility (Institute of Molecular Genetics, ASCR, v. v. i.). Estrogen-free food and water were supplied *ad libitum*. The total number of animals in the experiment was 24 in each group, sperm from both epididymides ($n=16$) of eight animals were analysed ($n=8$). Male mice were exposed to E_2 (Sigma) at a 20 ng/ml concentration either during puberty from of age (Pubertal E_2) or continuously from birth to 12 weeks of age (Continuous E_2). The control group of mice was not exposed to E_2. At the age of 12 weeks, the animals from all groups were killed by cervical dislocation. All animal procedures were carried out in strict accordance with the Animal Scientific Procedure, Art 2010, and approved by the Local Ethics Committee (approval number 151/2009).

Sperm preparation and capacitation

Mouse sperm cells were released from the distal regions of cauda epididymis into M2 fertilising medium (Sigma) under paraffin oil at 37 °C in 5% CO_2. Sperm viability and motility were checked (under a light microscope). Sperm stock was diluted to the required concentration (5×10^6/ml) into M2 medium under paraffin oil. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation *in vitro*. The time marked as 0 was the minimum time required for sperm to be added to the capacitated medium, removed from the medium and washed. This manipulation did not exceed 1 min. These samples served as a negative control. Sperm motility and viability were controlled at every experimental time point.

Immunofluorescent detection of TyrP

Samples of sperm cells were spread on microscope slides. After air-drying, sperm were fixed with 3.7% formaldehyde in PBS (pH 7.34) at room temperature for 10 min, followed by washing in PBS, incubated with 15 mM ammonium chloride (NH_4Cl)

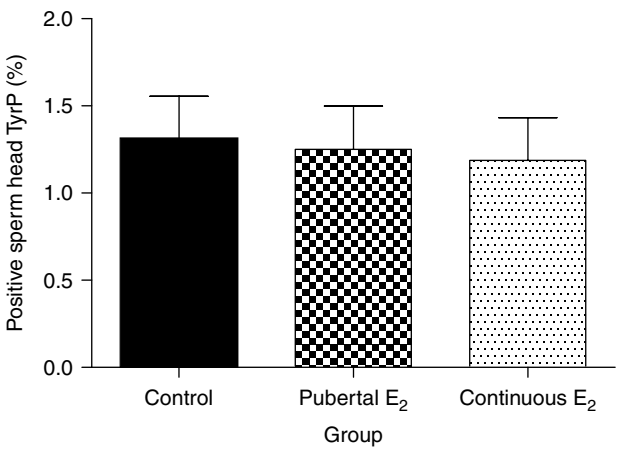

Figure 10 Number of TyrP-positive testicular sperm heads (%) among individual groups. Bars denote the arithmetical mean, whiskers denote S.E.M.

for 5 min, and with 0.1% Triton X-100 for 3 min. Slides were washed with PBS which was followed by immunofluorescent staining. Sperm were blocked with 10% BSA in PBS for 1 h and incubated with primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio, Prague, Czech Republic) diluted 1:500 in 1% BSA in PBS over night at 4 °C, followed by Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Prague, Czech Republic) secondary antibody 1:1000 in PBS for 1 h. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were examined with an epifluorescent microscope. For every experiment, we collected sperm data from eight mice. The positive or negative signal was evaluated from a total of 200 spermatozoa on every slide. In each group, at least two samples were analysed. Data were analysed statistically.

CTC fluorescent assay

Spermatozoa were resuspended in PBS and mixed with an equal volume (45/45 µl) of CTC solution (750 mmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris–HCl, pH 7.8) and incubated for 30 min. Cells were then fixed with 8 µl of 12.5% paraformaldehyde in 0.5 mol/l Tris–HCl (pH 7.4). After incubation, sperm suspension was smeared onto a glass slide and covered with a cover slip. To avoid evaporation and CTC fading, slides were kept in a wet chamber until evaluation. Samples were examined with a Nikon Labothot-2 fluorescent microscope equipped with a 40× Nikon Plan 40/0.65 and photographed with a COHU 4910 CCD camera (COHU Inc. Electronics Division, San Diego, USA) with LUCIA imaging software (Laboratory Imaging Ltd., Prague, Czech Republic).

SDS–PAGE with immunoblotting

SDS electrophoresis and immunoblotting technique was used for the TyrP assessment and was carried out using protocols based on standard methods (Laemmli 1970, Zigo *et al.* 2011). Suspension of non-capacitated sperm from a sperm stock released from cauda epididymis was used. Sperm samples were collected at 0, 30, 60, 90 and 120 min of capacitation *in vitro*, diluted with PBS and a final concentration of 10^6 sperm cells was ascertained using a Bürker chamber. Sperm pellets were resuspended in an equal volume of SDS–PAGE non-reduced sample buffer and heated at 97 °C for 3 min. Samples containing protein equivalent to 10^6 capacitated sperm cells were run on a 5% stacking and 10% running SDS polyacrylamide gel using Precision Plus Protein All Blue standards (Bio-Rad) as molecular weight markers. After transferring proteins onto a nitrocellulose membrane, non-specific sites were blocked with PBS blocking solution (5% skimmed milk and 0.05% Tween 20). Proteins phosphorylated on tyrosine residues were identified by the primary MAB anti-phosphotyrosine P-Tyr-01 (Exbio) diluted 1:500, followed by a peroxidase goat anti-mouse IgG secondary antibody (Sigma–Aldrich) diluted 1:20 000. Protein staining was visualised by chemiluminescence Super Signal West Dura (Thermo Scientific, Prague, Czech Republic). These experiments were performed at least three times with similar results. Representative results are shown.

Gel densitometry was performed with an Aida image analyser 4.18 (Raytest GmbH, Sprockhövel, Germany) and the relative intensity of individual signals was determined.

Quantitative RT-PCR (RTqPCR) of Tff1 *gene*

The analysis was performed according to the protocol from our previous study (Zatecka *et al.* 2013). The total RNA from one testis per animal was extracted by a Tri-Reagent Kit (Sigma) according to the manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity was measured on a spectrophotometer Helios A (Thermo Electron Corporation, Marietta, OH, USA). For the synthesis of cDNA, 5 µg purified RNA was used. Each sample was treated with 1 µl DNase I (Invitrogen), 1 µl DNase I reaction buffer (Fermentas, Burlington, ON, Canada) and H_2O to reach a volume of 10 µl. This mixture was incubated for 30 min at 37 °C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, NJ, USA). After incubation 1 µl EDTA (Fermentas) was added and followed by incubation at 65 °C for 10 min. Then 30 µl reaction mixture (8 µl reaction buffer for M-MuLV reverse transcriptase (Fermentas), 5 µl 10 mM 4dNTP (Fermentas), 0.3 µl RiboLock inhibitor (Fermentas), 1 µl oligo (dT) + random primers (Promega) and 15.2 µl H_2O were added to the samples. The mixture was incubated for 60 min at 42 °C followed by 10 min at 70 °C and at the end was maintained at 4 °C. Obtained cDNA was stored at −20 °C. For RT-qPCR – 5× diluted cDNA was used. The qPCR primer pair 5′-TGTCCGGGGATTCCCGTGGT-3′ (forward) and 5′-CCAGTGCCCAGGTGGAGGGT-3′ (reverse) specific for the mouse *Tff1* gene was used for RT-qPCR. The product length was 131 bp. For each reaction, 2 µl 5× diluted cDNA, 10 µl SYBR Green Master Mix (Fermentas), 0.5 µl primer and 7 µl H_2O were used. All reactions were performed in duplicates in a PCR cycler (Eppendorf, Prague, Czech Republic). The relative amount of mRNA in each sample was calculated from the measured quantification cycle (C_q) values. The expression of the reference gene for ribosomal 18S was used to normalise the measured values. The qPCR primer pair 5′-GTAACCCGTT-GAACCCCATT-3′ (forward) and 5′-CCATCCAATCGGTAG-TAGCG-3′ (reverse) with the product length 151 bp was used.

Measurement of the E_2 serum levels

Whole blood from mice was left for 60 min at room temperature. After incubation, the blood samples were centrifuged at 1200 ***g*** for 15 min. Serum levels of E_2 were analyzed using a RIA kit (Immunorad Beckman, Prague, Czech Republic). The detection limit was <2 pg/ml and the cross reactivity of antiserum with other serum estrogens was lower than 1%.

Preparation and analysis of testicular suspensions

Testes were placed into a glass homogeniser with 2 ml PBS and homogenised manually to obtain single-cell testicular suspension. After the homogenisation procedure the suspension was filtrated by a Cell Strainer 70 µm (BD Bioscience, Prague, Czech Republic) to remove residues of tough tissue. After

filtration, single-cell testicular suspension was centrifuged (300 ***g***), resuspended in 1 ml 4% formaldehyde in PBS (pH 7.0) and the cells were fixed at RT for 60 min. After fixation, the suspension was centrifuged at 300 ***g*** for 5 min, resuspended in 96% ethanol and refrigerated at −20 °C until the time of analysis. Samples of testicular suspensions were resuspended and washed 2× in PBS and spread on microscope slides. After this procedure, the protocol was the same as for the immunofluorescent detection of TyrP on epididymal sperm, but only 100 cells per slide were counted.

Statistical analysis

Experimental data were analysed using STATISTICA 6.0 (Statsoft, Prague, Czech Republic) and GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). The differences between the control and experimental groups in the number of the capacitated cells (TyrP positive sperm heads and CTC pattern B, Figs 2, 4, 6 and 7) were analysed by KW-ANOVA, and *post hoc* analysis was performed by Dunn's comparisons: $*P<0.05$, $**P<0.01$, $***P<0.001$. The calculated Pearson coefficients (r) were tested for their significance ($P\leq0.05$, Figs 8 and 9). The statistical power of the testicular sperm analysis was performed by STATISTICA 6.0 power module and was calculated for the ANOVA test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPLEMENT 4

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My contribution to this work: I was responsible for histological analysis and generated the experimental data using TUNEL assay, performed the statistical analysis of all data sets and helped to prepare the manuscript.

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Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice

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ABSTRACT

We tested the effect of two different concentrations (150 μg/l and 0.15 μg/l) of mycotoxin zearalenone (ZEA) on the reproductive parameters and expression of testicular genes in male mice. In adult males, no reduction of body or reproductive organ weight was observed, and the seminiferous tubules were morphologically normal with ongoing spermatogenesis. However, we found decreased sperm concentration, increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V. This study was also focused on the evaluation of gene expression profiles of 28 genes playing important roles during the processes occurring in the testicular tissue. We detected changes in the expression of genes important for proper spermatogenesis. Surprisingly, we observed a stronger effect after exposure to the lower dose of ZEA.

1. Introduction

Zearalenone (ZEA, F-2 toxin) is a nonsteroidal oestrogenic mycotoxin produced by a variety of *Fusarium* fungi, which are common contaminants of cereal crops worldwide [1]. ZEA is commonly found in maize or corns with the highest concentration in wheat, bran, corn and their products (*e.g.* corn flakes). ZEA is mainly a field contaminant; however the toxin production can also occur during storage in poor conditions [2]. Also, it has been shown that ZEA is transported from the fields to the aquatic systems by rain water [3].

The concentrations in food and feed vary over a wide range, depending on climatic conditions. Considering the mean levels of ZEA in the principal foods and their consumption, the average human daily intakes of ZEA range from 2.4 to 29 ng/kg b.w./day in adults, while toddlers (12–36 months old) have the highest average daily intakes ranging from 9.3 to 100 ng/kg b.w./day [2]. It has been shown that ZEA can also be excreted into cow milk [4].

ZEA is rapidly absorbed after oral administration. Its uptake is estimated to be approximately 80–85%, but it is difficult to measure owing to extensive biliary excretion. ZEA and its derivatives are detected in blood about 30 min after oral administration bound to human globulins as reproductive hormones [5,6]. Studies with radiolabelled zearalenone in mice showed that it is distributed to oestrogen target tissues such as uterus, interstitial cells of the testes and ovarian follicles. Some radiolabels were also found in adipose tissues, indicating that storage in adipose tissue may take place [7].

The main effect of zearalenone results from its oestrogenic activity. ZEA and its derivatives – α-zearanenol (α-ZOL) and β-zearalenol (β-ZOL) – compete with 17β-estradiol (E2) for the specific binding sites of oestrogen receptors (ERs). Several investigations have demonstrated that binding of ZEA and its derivatives initiates a sequence of events known to follow oestrogen stimulation [7]. Efficiency of binding of ZEA to ER in target tissues is <1–10% than that of E2, whereas α-ZOL shows stronger binding and β-ZOL lower affinity to ER [8]. The specific manifestations of ZEA are dependent upon the species, relative dose, and life stage during which ZEA is consumed. The most sensitive species is the pig; however it has been shown that ZEA can also have adverse effects on other species including rodents.

A study by Yang et al. [9] has shown that ZEA and α-ZOL affect steroidogenesis in mature mouse Leyidig cells *in vitro*. During this study authors observed a decrease of testosterone production in cells co-treated with ZEA or α-ZOL and human chorionic gonadotropin (hCG). They also detected decreased

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expression of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD-1), cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR), which play a crucial role during steroidogenesis. In adult animals testosterone is critical for proper spermatogenesis and sperm maturation, and disruption of spermatogenesis can thus adversely affect male fertility.

The negative effect of ZEA on reproductive parameters can also be observed *in vivo*. In another study of Yang et al. [10], adult male mice were exposed to intraperitoneal injections of ZEA or α-ZOL at the concentration 0, 25, 50 or 75 mg/kg b.w. daily for 7 days. In all groups the authors observed a significantly increased number of abnormal spermatozoa and significantly decreased number of live spermatozoa. Testicular and cauda epididymal sperm counts were also reduced, as well as serum testosterone. These effects were observed in the treated males at all doses in a dose-dependent manner. Besides the decrease in sperm quality, a significantly low pregnancy rate was observed when untreated females were mated with the treated males. At high concentrations (50 and 75 mg/kg b.w.), authors noticed a decrease of b.w. and increase of relative seminal vesicle weight.

To show whether the action of ZEA includes induction of apoptosis of testicular cells, Kim et al. [11] performed an *in vivo* study in rats. During this study 10-week-old male rats were exposed to a single intraperitoneal dose of ZEA (5 mg/kg b.w.) and analyzed at 3, 6, 12, 24, or 48 h after exposure. Germ cell degeneration caused by apoptosis was observed at stages I–VI of spermatogenesis 12 h after the exposure. The frequency of TUNEL-labelled germ cells increased in a stage-specific manner, with gradually increasing frequency at stages I–VI of seminiferous tubules with the time after exposure. These results show that a single dose of ZEA induces testicular germ cell apoptosis in a time-dependent and stage-specific manner in the rat testis *in vivo*. The induction of apoptosis in testicular tissue after ZEA treatment was also shown by Yuan et al. [12]; these authors have additionally shown that traditional medicinal plant *Gynostemma pentaphyllum* protects against toxicity caused by ZEA through anti-oxidation and anti-apoptosis effects mediated by the regulation of Bax and Bcl-2 expression.

Filipiak et al. [13] performed an *in vivo* study of pubertal rats in which they investigated the effect of two xenoestrogens, diethylstilbestrol (DES) and ZEA, with comparison of their effect with natural oestrogen 17β-estradiol (E2). While E2 and DES significantly reduced the numbers of spermatogonia, spermatocytes and Sertoli cells, ZEA only reduced the numbers of spermatogonia and Sertoli cells. The authors also measured the testis weight and seminiferous tubule diameter and length, which were significantly decreased by all three substances. In general, ZEA appeared to be the weakest of the three oestrogenic substances.

The aim of the present study was to assess the effect of treatment with a low dose of mycotoxin zearalenone on the male gonadal pathology, sperm quality and expression of selected genes. We have analyzed a wide range of genes expressed in the testes. For this purpose we selected genes playing important roles during spermatogenesis, genes expressed in Sertoli cells, and genes playing a role in apoptosis and hormonal response. We assume that analysis of these genes can reveal how ZEA affects germ cell development and subsequently the semen quality in mice.

2. Materials and methods

2.1. Animals and treatment, number of progeny, sex ratio

For our experiment we used the CD1 outbred mice strain (An Lab, Prague, Czech Republic) with high heterozygosity and average litter size (12–13 pups/litter). Mice (experimental and control groups) were kept under standard experimental conditions (constant temperature and 12-h light regime) in the animal facility of the Institute of Molecular Genetics, v. v. i., Prague. Animals were fed on soy-free feed (Ssniff, Soest, Germany). The diet and water were administered *ad libitum* and all stress factors were reduced to a minimum. Experimental groups were treated with different concentrations of ZEA (Sigma, Prague, Czech Republic), which was dissolved in drinking water. In this *in vivo* experiment there were two experimental groups – a group exposed to higher concentration of ZEA (150 μg/l), and a second group exposed to lower concentration of ZEA (0.15 μg/l). Animals exposed to the low dose were exposed to an environmentally relevant concentration (around 25 ng/kg b.w.) and animals exposed to the high dose were exposed to 1000 times higher concentration. ZEA was administered starting from the first day of mothers' pregnancy, and the number of progeny and the sex ratio were evaluated. The born pups were exposed during gestation, lactation, pre-pubertal and pubertal period, and up to the age of 70 days, when they were sacrificed and subjected to analysis. In each group, 18 animals were analyzed.

2.2. Anogenital distance, body and organ weight

Animals in the control and experimental groups were killed at the same age of 70 days. Anogenital distance (AGD) and body weight were measured and subsequently the reproductive organs were dissected and weighed. Left and right testes, epididymis, seminal vesicles and prostate were separated, cleaned and weighed individually. Immediately after weighing the left testes were frozen in liquid nitrogen and stored at −70 °C for further analysis; the right testes were used for histological analysis and epidymides were placed into warmed (37 °C) PBS for sperm release (see below).

2.3. Preparation of cells

Mouse sperm were obtained from the cauda epididymis. Spermatozoa were left to release spontaneously into 1 ml of warmed PBS at 37 °C in a CO_2 incubator for 15 min. Then the cell suspension was transferred into a new tube and PBS was added to 1 ml final volume. The concentration of spermatozoa was evaluated in a haemocytometer chamber under 100× magnification. Part of the epididymal spermatozoa was used for assessing viability, morphology and apoptotic stage. The rest of the suspension was washed twice in PBS, centrifuged for 15 min at 200 × *g* and dropped onto glass slides for immunocytochemical analysis.

2.4. Sperm viability

To determine the viability of epididymal spermatozoa a Live/Dead sperm viability kit was used (Invitrogen, Eugene, USA); the laboratory manual had to be slightly modified to enable its use for mouse sperm. First, the cell suspension was centrifuged for 5 min at 200 × *g*, then 1 μl of cell pellet was mixed with 200 μl of HEPES buffer and 1 μl 50× diluted SYBR 14 (in HEPES), and the mixture was incubated for 5 min at 37 °C in the dark. Subsequently, the mixture was centrifuged for 5 min at 200 × *g*. The supernatant was removed and the cell pellet was mixed with 200 μl of HEPES buffer and 1 μl propidium iodide (PI) and centrifuged for 5 min at 200 × *g*. The supernatant was removed and 20 μl of PBS was added, then 10 μl of the suspension was placed onto a glass slide and evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40× Nikon Plan Fluor 40/0.75 (Nikon, Prague, Czech Republic). Green (SYBR 14)-labelled spermatozoa were alive; orange-labelled (PI) spermatozoa were dead.

2.5. Sperm morphology

To evaluate the morphological state of spermatozoa, 10 μl of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled according to the protocol using Spermac Stain System (Ferti Pro, Beernem, Belgium). We evaluated at least 200 cells from each sample. Another method used to evaluate the sperm morphological status was indirect immunofluorescence with specific monoclonal antibody (Hs-14) against anti-acrosomal protein (see below).

2.6. Indirect immunofluorescence

Monoclonal antibody against intra-acrosomal protein (Hs-14) was used to assess the integrity of the acrosome. This antibody was prepared in our laboratory and is routinely used to test the acrosome state [14,15]. Epididymal spermatozoa were dropped on glass slides, air dried and fixed for 10 min with acetone. After rinsing with PBS, the slides were incubated overnight at 4 °C with monoclonal antibody (diluted to an immunoglobulin concentration 20 μg/ml). After thorough washing with PBS, the slides were incubated with anti-mouse IgM (μ-chain specific) fluorescein isothiocyanate (FITC) conjugate (Sigma, Prague, Czech Republic) diluted 1:128 in PBS and incubated for 60 min at 37 °C, washed with PBS and distilled water, and mounted in Vectashield H-1200 DAPI (Vector Laboratories, Burlingame, CA) for DNA visualization.

2.7. Apoptotic stage of spermatozoa

Apoptotic spermatozoa were detected using the APO-AF kit (Annexin V-FITC Apoptosis Detection Kit, Sigma, Prague, Czech Republic). The laboratory manual was slightly modified; 1 μl of cell suspension was mixed with 100 μl of 1× concentrated binding buffer and 1 μl of Annexin V solution. The mixture was incubated in the dark for 15 min at room temperature and centrifuged for 5 min at 200 × *g*. Supernatant was removed and 10 μl vectashield with DAPI was added. The mixture was placed onto a glass slide and examined with a Nikon Eclipse E400 fluorescent microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective and photographed with a CCD 1300-VDS camera (Vosskühler, Osnabrück, Germany) with the aid of the NIS-ELEMENTS AR imaging software (Laboratory Imaging, Prague, Czech Republic).

2.8. Histological analysis

The right testis was fixed in 4% formaldehyde in PBS for 48 h. Then the tissue was washed for 15 min in distilled water and dehydrated by increasing concentrations of ethanol. Subsequently, the tissue was placed in the mixture of acetone and xylene (1:1) for 30 min and then in xylene for 30 min. Finally, the testes were embedded in paraffin (Paraplast, Sigma, Prague, Czech Republic) and these paraffin blocks were cut in a microtome (sections 5 μm). For histological analysis the tissue sections were rehydrated by decreasing concentrations of ethanol and stained with haematoxylin-eosin. Tissue specimens were evaluated under a light microscope Olympus BX41 at 400× magnification and the images were photographed by camera Olympus IX81 (Olympus, Prague, Czech Republic).

2.9. TUNEL analysis

For detection of apoptotic cells in the paraffin sections of the testes, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method was employed, using an *in situ* detection kit (Promega, Madison, USA) according to the manufacturer's instructions. Samples were evaluated under a fluorescence microscope Nikon Eclipse E400, Nikon 40× Plan 40/0.65. Photographs were taken with a CCD camera VDS1300 and processed by NIS Elements AR software. In all specimens, the number of TUNEL-positive cells in 10 cross-sectioned seminiferous tubules was counted and six samples from each group were evaluated. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically analyzed.

2.10. RNA isolation

Total RNA was extracted from the left testis of experimental and control animals. For this extraction we used a Tri-Reagent kit (Sigma, Prague, Czech Republic). To each testis, 1 ml of Tri Reagent was added and the tissue was then homogenized in homogenizer Precellys 24 (Bertin Technologies, Aix-en-Provance, France). Subsequently, the samples were processed according to the manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity were measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA).

2.11. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For the synthesis of cDNA, 5 μg of purified RNA was used, to which we added 1 μl DNase I (Invitrogen, Eugene, USA), 1 μl DNase I reaction buffer (Fermentas, Burlington, Canada) and H_2O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37 °C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, USA). After incubation, 1 μl EDTA (Fermentas) was added and incubation continued at 65 °C for 10 min. Then 30 μl of the reaction mixture (8 μl of reaction buffer for M-MuLV reverse transcriptase (Fermentas, Burlington, Canada), 5 μl 10 mM 4dNTP (Fermentas, Burlington, Canada), 0.3 μl RiboLock inhibitor (Fermentas, Burlington, Canada), 1 μl oligo (dT) + random primers (Promega, Madison, USA) and 15.7 μl H_2O) was added to the samples. The mixture was incubated for 60 min at 42 °C, for 10 min at 70 °C, and in the end was maintained at 4 °C. The obtained cDNA was stored at −20 °C.

2.12. Quantitative polymerase chain reaction (qPCR)

Gene expression analysis was performed in BioMark (Fluidigm, San Francisco, CA), which enables performing a large number of real-time PCR reactions in a single run. Before performing BioMark analysis the samples were pre-amplified. The pre-amplification reaction was done as follows: 2 μl of cDNA (10 ng RNA/μl) was mixed with 1.2 μl of 208 nM primer mix (all primers were mixed together, final concentration of each primer 25 nM), 5 μl of iQ Supermix (BioRad, Prague, Czech Republic) and 1.8 μl of H_2O. The mixture was first incubated for 10 min at 95 °C, then followed 18 cycles of 15 s at 95 °C, and finally 4 min at 59 °C. Pre-amplified cDNA was diluted 20×. The real-time PCR reactions were carried out in GE Dynamic array 48.48 in a BioMark HD System (Fluidigm, San Francisco, California). Five μl of Fluidigm sample premix consisted of 1 μl of 20× diluted pre-amplified cDNA, 0.25 μl of 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), 2.5 μl of SsoFast EvaGreen Supermix (Bio-Rad, Czech Republic), 0.1 μl of 4× diluted ROX (Invitrogen, USA) and 1.15 μl of RNase/DNase-free water. Each 5 μl assay premix consisted of 2.5 μl of 10 μM primers (forward and reversed at a final concentration of 500 nM) and 2.5 μl of DA Assay Loading Reagent (Fluidigm, USA). Thermal conditions for qPCR were: 98 °C for 40 s, 40 cycles of 95 °C for 10 s, and 60 °C for 40 s. The β-actin (Actb) reference gene was selected from several reference gene candidates by Normfinder (GenEx Enterprise, MultiD Analyses, Sweden). The data were collected using BioMark 3.1.2 Data Collection software and analyzed by BioMark Real-Time PCR Analysis Software 3.1.3. (Fludigm, USA).

Table 1
Primer sequences.

Gene	Accession no.	Nucleotide sequence 5′–3′	Size of PCR product (bp)
Actb	NM_007393.3	CGGTTCCGATGCCCTGAGGCTCTT CGTCACACTTCATGATGGAATTGA	100
Ar	NM_013476.3	CCGGACCTTATGGGGACATGCG GCTGCCACAAGTGAGAGCTCCG	143
Bcl-2	NM_009741.3	CAGGGAGATGTCACCCCTGGTGG AGGCATCCCAGCCTCCGTTATCC	104
Ccnal	NM_007628.3	TTGCAGCTTGTCGGGACAGCA ACAAACTCATCCACGTCGGGCG	80
Ccndl	NM_007631.2	ACCTGGGCAGCCCCAACAAC GCCTGGCGCAGGCTTGACTC	139
Crem	NM_013498.2	GCCTCACCAGGAAG CCTGCAC TCTTCTTCCTGCGACACTCCCG	115
Ctsd	NM_009983.2	GACTCCCGGCGTCTTGCTGC AGCCGCCCACCTCCGTCATA	119
Dnmtl	NM_010066.4	AGCAAGTCGGACAGTGACACCCTTT GCCGAGTTCCCCTCTTCCGACT	149
Eps8	NM_007945.2	CCACTGCGGAGGAACGGAAGC CGTTGCGGAACCTCGGGACG	114
Fkbp5	NM_010220.3	GTTCGCAGAGCGGGACGCAA CTCCGTGGCGCAGCGTCATA	142
Fshr	NM_013523.3	GGAGCCTCTGGGCCAGTCGT GCGGTATGTTGACCTGGCCCTC	108
Grth	NM_013932.4	TACTTTGGGGAGGCGACGCC AGGTTCTTCCTGGGGTGGACGA	85
Igfbp5	NM_010518.2	CGCGGGGTTTGCCTCAACGA GGCCGGAAGACCTTGGGGGA	128
Icap1	NM_008403.4	GCCGCTGGACCATCCCACAC GCTCGATCCAGAGAAGATGCCGC	111
Kdm4a	NM_172382.2	GCAAG CTCCCACG CCACCAC CCTCGGGG GTCAGCTGCTCA	81
Mas1	NM_008552.4	CCGAGACTGCCCCAAGCCTCT TGCCCTGGGTCACTTCAGGTCAT	108
Meig1	NM_008579.4	TCTGCACTGAGTCTGGTCGTCGA TCCTCTGACCATTTCTTGGCACGA	144
p21	NM_007669.4	CCGCCGCGGTGTCAGAGTCTA CTGTGCGGAACAGGTCGGAC	120
p53	NM_001127233.1	ATGGCTTCCACCTGG GCTTCCTG CCACAACTGCACAGGGCACGT	119
Sox9	NM_011448.4	GCTGGAAGTCGGAGAGCCGAGA AGAGAACGAAACCGGGGCCAC	137
Spata2	NM_170756.2	GGGCCTGTGCTTTGGAGGCG TGGCTCTGGAACTGGAGGCTGG	115
Sycpl	NM_011516.2	GCCCATGCTCGAACAG GTTGC ACAGTCTGCTCATTGGCTCTGAA	98
Sycp3	NM_011517.2	GGACAGCGACAGCTCACCGG GGTGGCTTCCCAGATTTCCCAGA	90
Tffl	NM_009362.2	TGTCCGGGGATTCCCGTGGT CCAGTGCCCAGGTGGAGGGT	131
Tnpl	NM_009407.2	CCGAGCTCCTCACAAG GGCGT CAGGGCAGAGCTCATTGCCGC	140
Tnp2	NM_013694.4	CCTGCAAGACCCAGCCACCG GTTTCCGCCTCCTGACGGCC	94
Vegfa	NM_001025257.3	TGCTCTCTTGGGTGCACTGGAC GACGGCAGTAGCTTCGCTGGT	147
Wtl	NM_144783.2	GGCGCTTTGAGGGGTCCGAC AAAGTGGGCGGAGCACCGAC	205

Table 2
Effect of ZE on body and organ parameters in CD1 mice. Mean ± SD; $n = 18$.

Group	AGD (cm)	Body weight (g)	Testes (g)	Prostate (g)	Seminal vesicles (g)	Epididymis (g)
Control	1.57 ± 0.15	35.10 ± 2.53	0.261 ± 0.030	0.159 ± 0.030	0.310 ± 0.052	0.088 ± 0.010
High dose	1.53 ± 0.12	33.81 ± 4.30	0.239 ± 0.022	0.156 ± 0.036	0.291 ± 0.066	0.084 ± 0.006
Low dose	1.48 ± 0.07	34.63 ± 2.36	0.248 ± 0.023	0.151 ± 0.036	0.309 ± 0.041	0.087 ± 0.007

The cut-off value for Cq was set at 25 and values higher than that were replaced by the Cq value of 25. The missing data were filled with maximum of a column plus 1. Data were normalized with β-actin. The fold change in expression was calculated using the $2^{-\Delta\Delta Cq}$ method [16] for each sample and then expressed as the mean of all these fold changes. The control was set at 100% and experimental samples were compared to the control.

2.13. Selected testicular genes

Twenty-eight genes that are expressed in testicular tissue, and thus can influence the process of spermatogenesis and consequently the sperm quality, were tested. The tested genes can be divided into five groups – genes expressed in the germinal cell line (*Vegfa*, *Sycp3*, *Sycp1*, *Ccna1*, *Meig1*, *Grth*, *Prm1*, *Tnp1*, *Tnp2*), genes expressed in Sertoli cells (*Sox9*, *Wt1*, *Eps8*, *Icap1*, *Mas1*), genes playing a role in hormonal response (*Ar*, *Fkbp5*, *Tff1*, *Igfbp5*, *Ctsd*, *Fshr*), genes playing a role during apoptosis (*p21*, *Bcl*, *p53*), and genes related to epigenetic processes (*Ccnd1*, *Crem*, *Kdm4a*, *Spata2*, *Dnmt1*). Primer properties are summarized in Table 1.

2.14. Statistical analysis

Statistical analysis was performed in STATISTICA 7.0 (StatSoft, Prague, Czech Republic). The number of offspring was analyzed by the Mann–Whitney *U*-test. The body weight was compared using the analysis of variance (ANOVA), and *post hoc* analysis was performed using the Newman–Keuls test. The weights of individual organs were compared using the analysis of covariance (ANCOVA) and the body weight was used as a covariate. Data obtained from the sperm analysis and TUNEL method were analyzed using the Kruskal–Wallis ANOVA; *post hoc* analysis was performed again using Dunn's test. Statistical analysis of gene expression was performed in Genex 5.3.7 (MultiD Analyses, Sweden) using one-way ANOVA, and *post hoc* analysis was performed using the Dunnett test. A *P*-value lower than 0.05 was identified as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Number of progeny; sex ratio; body and organ weight

Male mice were exposed to two different concentrations of ZEA during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood. To evaluate the effect of ZEA on the male reproductive tract, the body and organ weight, number and sex ratio of progeny, and anogenital distance (AGD) were measured. Individuals from experimental groups were compared with control animals. This model was used in all subsequent measurements. ZEA had no effect on the number of progeny and sex ratio in both experimental groups (not shown). AGD and body weight as well as the weight of reproductive organs were not affected in these two groups (Table 2).

3.2. Sperm parameters

To evaluate the effect of ZEA on sperm quality, the sperm morphology (Fig. 1), viability and state of acrosome were evaluated. It was shown that ZEA induced a decrease in sperm quality mainly in animals exposed to the low dose of ZEA. In this group we detected significantly decreased sperm concentration (by 40%) and increased number of morphologically abnormal spermatozoa. Moreover, a significantly increased number of apoptotic spermatozoa and changes of acrosome staining were observed in this group. However, the sperm parameters of animals exposed to the high dose were also affected. We detected an increased number of apoptotic spermatozoa and changes in acrosome staining in samples obtained from this group (Table 3).

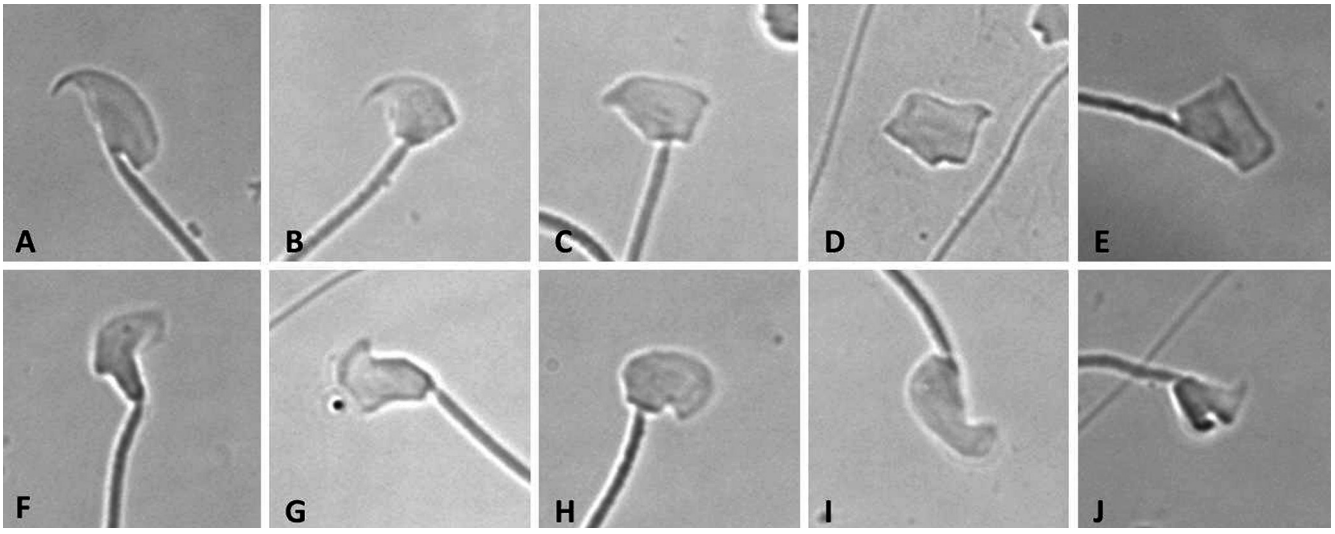

Fig. 1. Exemplary pictures from light microscopy showing sperm with normal (A) and aberrant morphology (B–J).

Table 3
Effect of ZE on sperm parameters of CD1 mice. The sperm status was analyzed by monoclonal antibodies against intra-acrosomal sperm protein (pHs-14), Hoechst 33342 (viability) and Annexin V kits. The mixture of sperm suspension was smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on each glass slide. Mean ± SD; $n = 18$.

Group	Sperm concentration ($\times 10^6$/ml)	Sperm morphology (% of normal cells)	Sperm viability (% of live cells)	Annexin V (% of positive cells)	HS-14 (% of acrosome positive cells)
Control	38.70 ± 9.35	93.60 ± 1.60	91.00 ± 1.41	61.00 ± 2.45	72.10 ± 1.96
High dose	29.90 ± 11.71	91.25 ± 3.02	89.80 ± 1.17	70.50 ± 2.26***	76.60 ± 3.58**
Low dose	23.80 ± 9.75*	91.20 ± 1.93*	89.40 ± 1.34	84.00 ± 3.16***	76.40 ± 3.98**

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3.3. Histology of testes

Histological analysis was performed with testis paraffin sections. Morphology of seminiferous tubules and the process of spermatogenesis were evaluated visually under a microscope. In experimental groups no pathological patterns in the morphology of seminiferous tubules were observed compared to the control. Also, the process of spermatogenesis was not interrupted. In all groups, spermatogonia, spermatocytes and prolonged spermatides released into the lumen of seminiferous tubules were visible. During the examination of histological slides we did not observe any statistically significant differences in the absolute number of appropriate cell types between control and experimental groups. The group exposed to the high dose had a higher number of Sertoli cells and lower number of spermatogonia and spermatocytes compared to the control, but the differences were not significant (data not shown).

3.4. TUNEL analysis

TUNEL analysis was used to detect apoptotic cells in the testis paraffin sections. In the group exposed to the low dose we observed a slightly higher incidence of apoptotic cells, but the values did not reach the level of significance (Fig. 2). TUNEL-positive cells were observed mainly in the basal lamina of seminiferous tubules.

3.5. Real-time PCR of testicular genes

Genetic analysis was performed by quantitative Reverse Transcription Polymerase Chain Reaction (qPCR), which was performed using BioMark. The list of the tested genes (see Section 2.13) is given in Table 1.

Our experiments showed changes in the expression pattern of germinal cell-specific genes mainly in group, which was exposed to the lower concentration of ZEA. In this group we observed significantly decreased expression of the *Vegfa* gene, which is expressed specifically in spermatogonial cells. We also detected decreased expression of several genes specific for spermatocytes – *Sycp3*, *Ccna1*, and *Grth* and increased expression of gene *Sycp1*, which is also expressed in spermatocytes. Expression of the genes specific for round spermatids was not changed. In group exposed to the high dose we did not detect any significant changes in the expression of the tested germ-cell genes. The results are summarized in Fig. 3.

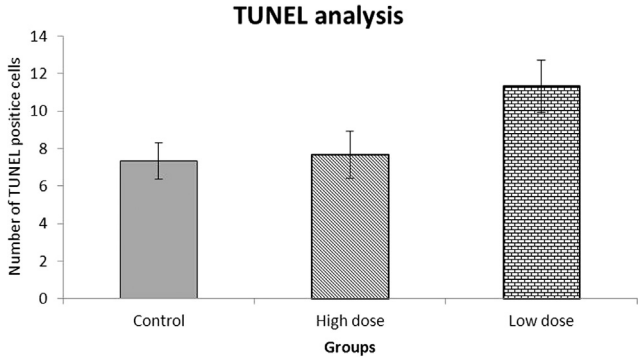

Fig. 2. Number of TUNEL-positive cells in testicular tissue sections. The high-dose group was exposed to 150 μg/l of ZE; the low-dose group was exposed to 0.15 μg/l of ZE. Mean ± SD; $n = 18$.

In the case of genes expressed specifically in Sertoli cells, we detected significantly decreased expression of *Sox9* genes in both groups and also decreased expression of *Wt1* genes in the group exposed to the high dose. In the group exposed to the low dose we detected increased expression of the *Mas1* gene. Two other tested genes (*Eps8* and *Icap1*) did not reveal any significant changes in gene expression (Fig. 4).

We detected decreased expression of the gene for androgen receptor (*Ar*) and its component *Fkbp5*. Expression of the genes playing a role in the response to oestrogen stimuli (*Tff1*, *Igfbp5* and *Ctsd*) was not changed. Expression of the gene for follicle-stimulating hormone receptor (*Fshr*) was not significantly changed (Fig. 5).

Among the tested apoptotic genes we found two genes with decreased expression, *p53* and *p21*; the decrease was observed in both experimental groups. Expression of the *Bcl2* gene was not changed (Fig. 6).

In the case of genes related to epigenetic processes, we detected significantly decreased expression of genes for Ccnd1 and Dnmt1 in the animals exposed to the low dose of ZEA and significantly decreased expression of genes for Ccnd1, Kdm4a and Spata2 in animals from group exposed to the high dose (Fig. 7).

4. Discussion

The aim of this study was to describe the influence of the mycotoxin zearalenone on the reproductive parameters and expression of selected genes in CD1 outbred mice. The tested reproductive parameters included the number of progeny in the parental generation, body and organ weight, anogenital distance, sperm quality, histopathology of the testes, apoptotic stage of testicular cells, and the expression profile of testicular genes in the offspring. Experimental animals were exposed to two different concentrations of ZEA, which was administered in drinking water. One group was exposed to the concentration of 150 μg/l and the other group to 1000 times lower concentration – 0.15 μg/l. The control group was not exposed at all. The average daily dose of ZEA for an adult man ranges from 2.4 to 29 ng/kg b.w. [2], which corresponds to our low dose (~25 ng/kg b.w.). Experimental animals were exposed starting from the first day of mothers' pregnancy up to the age of 70 days, when the animals were killed and subjected to analysis.

Our work is unique because, compared to other studies, it is focused on the effect of a low concentration of ZEA. The low concentration that was used in our experiment corresponds to the average concentration to which is exposed human population.

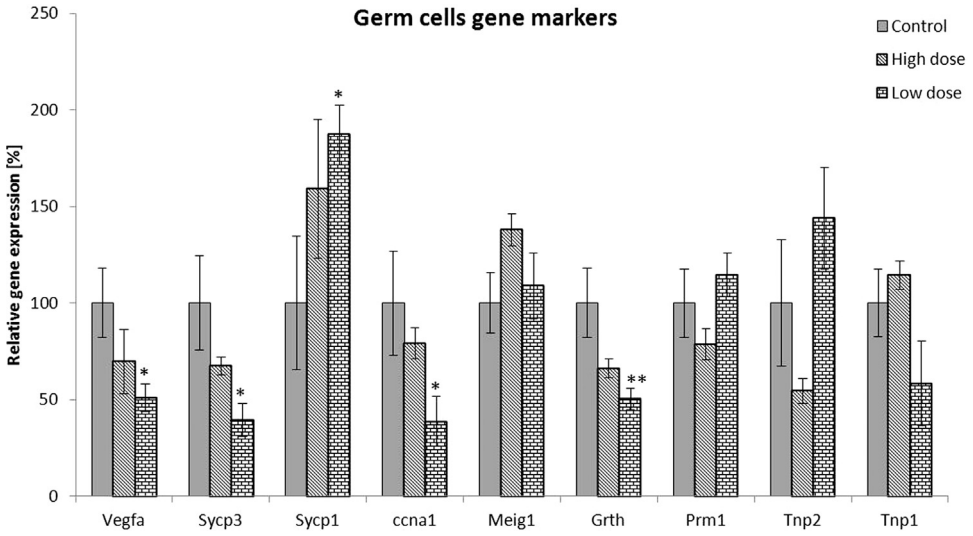

Fig. 3. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in testicular germ cells. In this study, genes for vascular endothelial growth factor (*Vegfa*), synaptonemal complex protein 3 (*Sycp3*), synaptonemal complex protein 1 (*Sycp1*), cyclin A1 (*Ccna1*), meiosis expressed gene 1 (*Meig1*), DEAD box polypeptide 25 (*Grth*), protamine 1 (*Prm1*), transition protein 2 (*Tnp2*) and transition protein 1 (*Tnp1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SE; $n = 9$.

Animals exposed *in utero* were born with no observable pathologies; the number or sex ratio of the progeny was not affected in both concentration groups. In adult males we did not observe reduced body or reproductive organ weight, and the seminiferous tubules were morphologically normal with ongoing spermatogenesis. The first effect was observed during the evaluation of sperm parameters. We detected decreased sperm concentration, increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V in the low-dose group. The decreased sperm quality and sperm count was also observed in the study by Yang et al. [10] after daily intraperitoneal injections of ZEA given for 7 days. It should be noted that the decrease in sperm concentration in the group exposed to the low concentration was almost 40%. Nevertheless, an initial concentration of about 38.7 million sperm/ml is still sufficient for reproduction. However, in case of human population where low quality of human sperm is observed, ZEA can have a significant effect on human fertility. Since the TUNEL method did not reveal any increase of apoptotic cells in the testes, and the expression of apoptotic genes was not changed in this group, it can be assumed that the reduction in the number of mature spermatozoa originated already at the level of spermatogonia. This would be in agreement with the decreased expression of the spermatogonial *Vegfa* gene (Fig. 3). The decreased number of spermatogonia after ZEA treatment was also found in the study by Filipiak et al. [13] in pubertal rats. On the other hand, two other studies have shown that ZEA is able to induce apoptosis in testicular tissue of the exposed animals [11,12]; however, in these studies much higher concentration of ZEA or different type of exposure was used.

Besides evaluation of reproductive parameters, we focused on the detection of changes in gene expression. It has been shown previously that ZEA is able to significantly influence the expression of testicular genes and thus negatively affect spermatogenesis [17]. In total we evaluated 28 genes. These genes were divided into five groups according to their function.

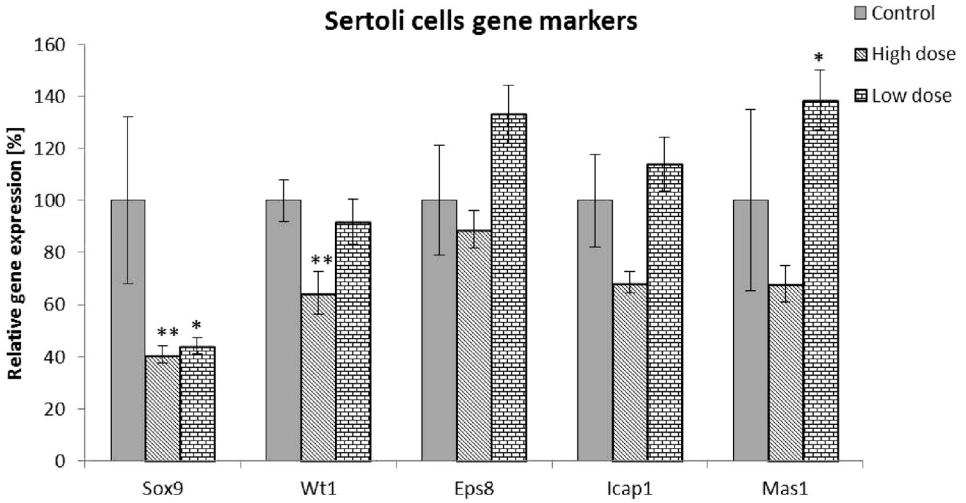

Fig. 4. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes expressed in Sertoli cells. In this study, genes for SRY-box containing gene 9 (*Sox9*), Wilms tumour 1 (*Wt1*), epidermal growth factor receptor pathway substrate 8 (*Eps8*), integrin beta 1 binding protein 1 (*Icap1*) and MAS1 oncogene (*Mas1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SE; $n = 9$.

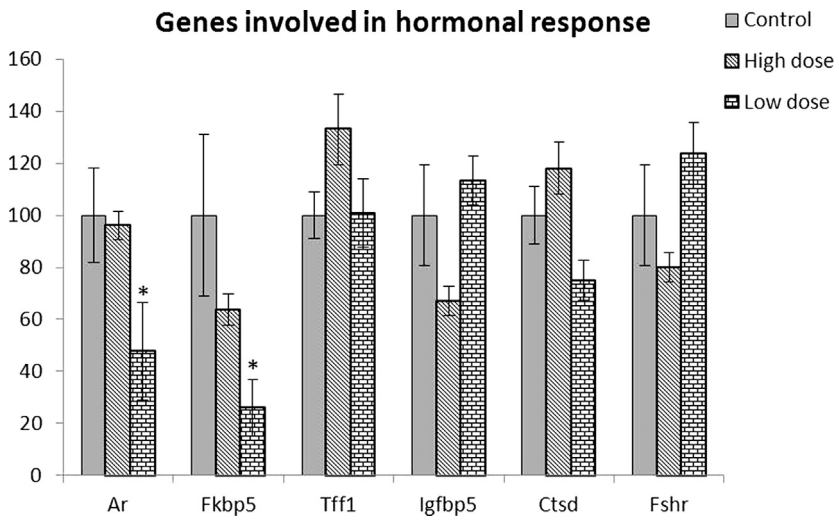

Fig. 5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in the response to various hormonal stimuli. In this study, genes for androgen receptor (*Ar*), FK506 binding protein 5 (*Fkbp5*), trefoil factor 1 (*Tff1*), insulin-like growth factor binding protein 5 (*Igfbp5*), cathepsin D (*Ctsd*) and follicle-stimulating hormone receptor (*Fshr*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$. Mean ± SE; $n = 9$.

The first group included genes regulating apoptosis – *p53*, *p21* and *Bcl2* (Fig. 6). p53 is an evolutionarily very conserved tumour suppressor protein that regulates the cell cycle, DNA repair, and apoptosis and it is also a transcription factor for *p21* and *Bax* genes. In this study we detected decreased expression of genes for p53 and p21 proteins in both experimental groups, although in the group exposed to the low dose the effect was slightly more noticeable. Since the expression of *p53* and *p21* is interrelated, it is not surprising that the change in their expression was nearly identical. The fact that these genes were downregulated indicates that ZEA had probably no effect on DNA damage. Expression of the gene encoding the antiapoptotic protein Bcl-2 was not changed, and our previous results (TUNEL assay) also did not show any changes in the number of apoptotic cells in testicular tissue (Fig. 2). A study made in fish *Kryptolebias marmoratus* has shown that short-term exposure to various endocrine disruptors (ED) such as bisphenol A or 4-nonylphenol increased *p53* expression, but after long-term exposure the expression of *p53* declined. The authors suggest that this may lead to an increased risk of potential cancer development [18].

Among further tested genes were genes expressed in testicular germ cells. The gene for protein Vegfa is expressed in spermatogonial cells and it plays a role during self-renewal and differentiation of these cells [19]. It has been shown that *in vitro* treatment of bovine testicular tissue with Vegfa results in significantly more differentiating germ cells in bovine testis and conversely, blocking Vegfa activity leads to significantly reduced numbers of germ cells [20]. Vegfa thus may support germ cell survival and sperm production. We detected decreased expression of this gene in the group exposed to the low dose of ZEA. We also tested expression of the genes specific for spermatocytes – *Sycp1*, *Sycp3*, *Ccna1*, *Meig1* and *Grth*. Sycp1 and Sycp3 are the main components of synaptonemal complex (SC) – structure playing a crucial role in synapsis and recombination during meiosis. After exposure to the low concentration of ZEA the expression of *Sycp3* decreased by almost 50% and conversely, the expression of *Sycp1* increased to 206% compared to

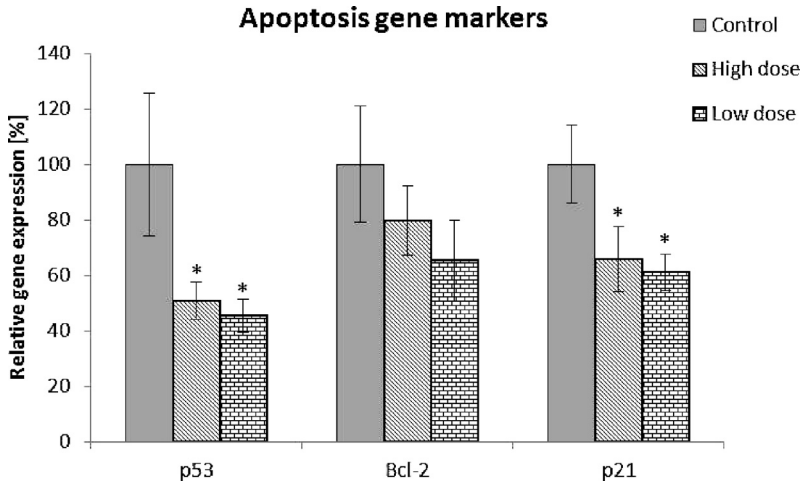

Fig. 6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes playing a role in apoptosis. In this study, genes for tumour protein p53 (*p53*), B-cell leukaemia/lymphoma 2 (*Bcl2*) and cyclin-dependent kinase inhibitor 1A (*p21*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control group. Whiskers indicate the standard error. $^{*}P < 0.05$. Mean ± SE; $n = 9$.

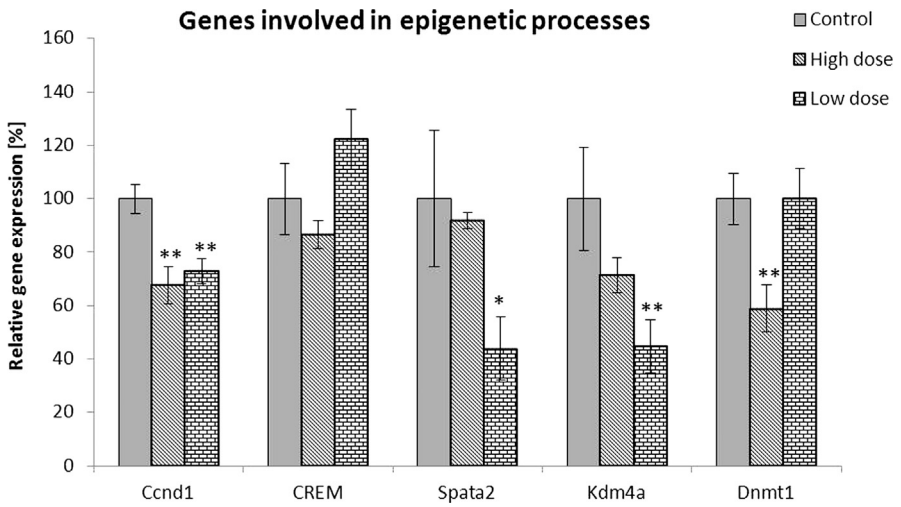

Fig. 7. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of genes that are related to epigenetic processes. In this study, genes for cyclin D1 (*Ccnd1*), cAMP responsive element modulator (*Crem*), lysine (K)-specific demethylase 4A (*Kdm4a*), spermatogenesis associated 2 (*Spata2*) and DNA methyltransferase (cytosine-5) 1 (*Dnmt1*) were tested. The graph illustrates the relative expression of the selected genes. The control group represents 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups and the control groups Whiskers indicate the standard error. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SE; $n = 9$.

the control. However, it is unlikely that these two proteins could functionally substitute for each other because they have different functions – Sycp1 forms transverse filaments while Sycp3 forms lateral filaments of SC [21]. Recent studies have shown that Sycp1 and Sycp3 are also required for centromere pairing in the mouse, which is important for proper chromosome segregation [22]. Errors in meiotic chromosome segregation are the main cause of human aneuploidy, and therefore their altered expression, especially lack of Sycp3, could have a negative effect on the sperm quality. In the group exposed to the low dose we also detected about 50% decreased expression of cyclin A1 (*Ccna1*) compared to control. Cyclins are proteins regulating the eukaryotic cell cycle. *Ccna1* is expressed in meiotic cells and it has been shown that it is essential for spermatocytes to go through the first meiotic division. *Ccna1*$^{-/-}$ mice were sterile due to the block of spermatogenesis before the first meiotic division [23]. Mice with heterozygous mutation (*Ccna1*$^{+/-}$) were subfertile because of a low sperm count. *Ccna1*$^{+/-}$ male mice had about half the cyclin A1 protein compared to normal mice. All types of spermatogenic lineage cells were present, but the number of haploid spermatids and spermatozoa was significantly lower [24]. We can therefore say that the phenotype of *Ccna1*$^{+/-}$ mice is similar to the phenotype observed in the animals exposed to the low dose of ZEA, in which we detected about half the level of Ccna1 mRNA and nearly 40% fewer spermatozoa compared to the control, but at the same time we were able to observe ongoing spermatogenesis. We also detected decreased expression of testis-specific gonadotropin-regulated RNA helicase (*Grth*) in the testes of animals from the low-dose group. *Grth* is expressed in Leydig and germ cells (spermatocytes and round spermatids) and it is target of gonadotropin and androgen action. *Grth* knockout mice (*Grth*$^{-/-}$) suffer from azoospermia caused by spermiogenesis failure. *Grth* is an mRNA-binding protein that controls translation of mRNA whose proteins are expressed in different phases of spermiogenesis, *e.g.* transition proteins or protamines [25]. In our study we did not detect any changes of gene expression of transition proteins 1 and 2 or protamine 2 either; however, we have not tested the protein level and therefore we cannot say whether the translation was affected or not. Nevertheless, the decreased expression of *Grth* could contribute to the decreased sperm quality of animals exposed to the low dose. Taken together, it seems that ZEA may influence the spermatogonial cells – their differentiation and self-renewal – and also the spermatocytes by affecting expression of several important meiotic genes. This might be the reason for the decreased number of epididymal spermatozoa and their decreased quality in animals exposed to the low dose.

To assess the potential effect of ZEA on Sertoli cells we examined the expression profile of several Sertoli cell-specific genes (Fig. 4). One of them was nuclear transcription factor Sox9, which is preferentially expressed in Sertoli cells. In the testis of fertile one-year-old rats the Sertoli cells of some tubule segments were strongly positive for *Sox9* whereas other were negative, and further studies showed that the reaction varied systematically according to the zone or stage of spermatogenesis. In general, it is possible to say that a prominent presence of *Sox9* in the Sertoli cells is related to abundant mitosis and meiosis [26]. Decreased expression of the *Sox9* gene was detected in both groups. This effect was more obvious in the group exposed to the high dose (reduced by 38%) than in the group with low exposure (reduced by 32%) and could be caused by decreased spermatogenesis in these groups. We also analyzed expression of transcription factor *Wt1*, which is specific for foetal and adult Sertoli cells. Disruption of *Wt1* expression in adult Sertoli cells leads to interruption of apical ectoplasmic specialization (ES) (junctional complex between Sertoli cells and elongating spermatids). This can cause increased germ-cell apoptosis and reduced sperm count or motility. In mice with impaired expression of *Wt1* in Sertoli cells, dysregulation of several other genes was observed. Among these were genes for *Eps8* and *Icap1-α*, which are important signalling molecules in apical ES [27]. We detected decreased expression of the *Wt1* gene in the group exposed to the low dose (reduced by 28%); however, we did not detect any significant changes in the expression of *Eps8* and *Icap1a* genes. We therefore assume that apical ES were not substantially affected in the testes of experimental animals. Another tested gene whose expression was increased in the group exposed to the low dose was *Mas1*. *Mas1* is a G-protein-coupled receptor which is expressed in Sertoli and Leydig cells. Studies of *Mas*-knockout mice showed that lack of Mas1 affects the expression of enzymes involved in the biosynthesis of testosterone in Leydig cells. This suggests a possible role of *Mas1* in the regulation of androgen metabolism in the male reproductive system [28].

Another group of tested genes included the genes that are somehow connected with epigenetics (Fig. 7). One of them was DNA methyltransferase 1 – *Dnmt1*. *Dnmt1* has preference for hemimethylated DNA and thus is critical for maintaining

the methylation pattern during DNA replication. The protein is localized in the nuclei of all male germ cells up to pachytene spermatocytes and it is important for proper spermatogenesis [29]. We detected decreased expression of this gene in animals exposed to the high dose of ZEA. Another tested gene was the gene for lysine-specific demethylase 4A (*Kdm4a*), which specifically demethylates histone H3K9. Its expression is restricted to post-meiotic male germ cells and it is highest in round spermatids. In *Kdm4a*-deficient mice, chromatin condensation defects were observed and it has also been shown that *Kdm4a* directly binds to *Tnp1* and *Prm1* genes and thus controls their expression [30]. It has also been reported that Kdm4a interacts directly with androgen receptor (Ar) and is involved in transcription activation of target genes [31]. We detected significantly decreased expression of *Kdm4a* in the group exposed to the low dose. However, neither *Prm1* nor *Tnp1* expression was changed in this group and on the other hand, the expression of *Ar* was decreased. Here, we can only speculate whether the decreased expression of androgen receptor was linked with decreased expression of *Kdm4a* or whether it had another cause. We also tested the expression of spermatogenesis-associated protein 2 (*Spata2*). *Spata2* is expressed in Sertoli cells, where its expression is developmentally regulated. Its expression is stimulated by FSH and might also be regulated by methylation of its promoter [32]. We detected decreased expression of this gene in the group exposed to the low dose. The last tested gene was *Ccnd1* – G1/S-specific cyclin D1. Expression of the cyclin D1 gene is regulated by methylation of its promoter. Ccnd1 together with cyclin-dependent kinases 4 and 6 regulate the cell cycle transition from G1 to S phase [33]. We detected reduced expression of this gene in both treated groups. It is hypothesized that exposure to environmental pollutants may induce epigenetic changes. For example, a study of Stouder and Paolini-Giacobino [34] showed that endocrine disruptor vinclozoline is able to induce some epigenetic changes. In this study alterations in the imprinting of five paternally/maternally imprinted genes in the sperm of the offspring after administration of vinclozoline to pregnant female mice were observed. Another study showed an association between imprinting errors and azoospermia [35]. These findings indicate that epigenetics could be one of the mechanism by which the endocrine disruptors act.

We have also analyzed expression of the genes playing a role in the response to androgen (*Ar*, *Fkbp5*) and oestrogen (*Tff1*, *Igfbp5*, *Ctsd*) stimuli. In the group exposed to the low dose, we detected decreased expression of the gene for androgen receptor (*Ar*) and its component *Fkbp5*. Contrary to that, we did not detect any changes in the expression of oestrogen-responsive genes. In general, zearalenone is considered to be a substance with oestrogenic activity. This has been shown in many studies *in vitro* as well as *in vivo*. Oestrogenic activity *in vitro* has been proven by E-screen assays on MCF-7 cells [36]. In another study, authors elegantly demonstrated that ZEA acts as a full agonist to ERα-mediated transcription and a mixed agonist/antagonist for ERβ [37]. The effect of ZEA in female laboratory animals such as mice [38,39], rats [40] or pigs [41] also demonstrates its oestrogenic activity. The *in vivo* effect of ZEA on male mice is not so clear. Several studies have shown that ZEA influences the testosterone level *in vitro* [9] as well as *in vivo* [10]. Moreover, it has been demonstrated that *in utero* exposure to ZEA results in increased foetal anogenital distance, indicating an androgenic effect during foetal development [42]. In our study we did not observe any noticeable oestrogen effect of ZEA on male mice, but on the other hand we detected decreased expression of *Ar* and its component Fkbp5. Taken together with the increased expression of the *Mas1* gene and decreased expression of the *Grth* gene, we can assume that during our study ZEA also had a certain effect on the androgen hormonal system. Moreover, this idea is supported by previous studies on specific localization of ZEA to the site of testosterone synthesis in the interstitial region of the testes [43].

Animals exposed to the low ZEA concentration were affected considerably more than animals exposed to the high ZEA concentration. A similar result was observed in our laboratory for endocrine disruptor bisphenol A (BPA); the lower dose had a greater negative impact on the reproduction parameters and fertilization of mice *in vivo* [14]. BPA has been used as a model for the low dose effect and non-monotonic dose-response curve studies of endocrine disrupting chemicals. It was shown to directly bind to ER, albeit with lower affinity than natural oestrogen. It is worth noting that BPA showed both antiestrogenic and antiandrogenic effects and interfered with thyroid hormone action [44,45].

This phenomenon, when lower dosages of hormones have some effect while the high dosages have no or opposite effect, is quite common in endocrinology. There are several explanations for this phenomenon. The hormonally active endocrine disruptors interfere with endogenous hormones and act through the same mechanism, which means they bind to the receptors that subsequently mediate the response which depends on ligand concentration [46]. Also, it has been shown that at concentrations exceeding its physiological value the ligand may bind to receptors for a different hormone and induce the response [47]. Apparently different responses may therefore be observed depending on the hormone concentration.

5. Conclusion

Our study showed that a low concentration of mycotoxin zearalenone is able to negatively influence the sperm parameters and testicular gene expression of CD1 mice *in vivo*. In experimental animals we detected decreased sperm concentration (by 40%) and impairment of sperm quality (morphology and increase of apoptotic spermatozoa). Based on our experiments we can assume that the decrease in sperm concentration has its origin at the level of spermatogonia. The meiotic phase of spermatogenesis was affected by ZEA as well, and this could have caused further decrease of sperm quality. Our results have also shown that the lower dose of ZEA had a greater effect on the sperm quality and expression of important testicular genes. One possible explanation could be that zearalenone acts at the hormonal level, and it is known that a low concentration of a hormone may act stimulatingly, while high concentrations may have an opposite or no effect.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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SUPPLEMENT 5

Zatecka, E, L Ded, F Elzeinova, A Kubatova, A Dorosh, H Margaryan, P Dostalova, and J Peknicova 2013 Effect of tetrabrombisphenol A on induction of apoptosis in the testes and changes in expression of selected testicular genes in CD1 mice. *Reproductive Toxicology* **35** 32-39

My contribution to this work: I was responsible for histological analysis and generated the experimental data using TUNEL assay, performed the statistical analysis of all data sets and helped to prepare the manuscript.

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Effect of tetrabrombisphenol A on induction of apoptosis in the testes and changes in expression of selected testicular genes in CD1 mice

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is a substance widely used in industry as a flame retardant. TBBPA was found in the environment and was detected even in the human body. The effect of this chemical was observed in different cell lines in vitro and it is supposed that TBBPA may affect various hormonal systems in vivo. In this study we examined the effect of TBBPA on the reproductive parameters of two generations of outbred mice in vivo. Experimental and control animals of F1 generation were bred in various conditions to enable evaluation of the possible trans-generational effect. An increased incidence of apoptosis in the testes and changes in the morphometry of seminiferous tubules was detected in the experimental animals. In addition, changes in the expression pattern of selected genes encoding proteins that play an important role during spermatogenesis were observed. In contrast, sperm quality and reproduction were not affected by TBBPA.

1. Introduction

Every day, numerous environmental pollutants get into our environment. These compounds, which are not naturally present in nature, can harmfully influence wildlife and the human population. Many of them can act as so-called endocrine disruptors which can disturb the physiologic function of endogenous hormones [1]. Frequently, they act as weak oestrogenic, antioestrogenic or antiandrogenic compounds, and it has been shown that they can affect both male and female reproductive development and function [2–5].

An inherent group of these compounds is represented by flame retardants. The most important and widely used group of flame retardants are brominated flame retardants (BFRs) [6]. Their production is steadily increasing [7]. Due to their structure and bromide substituents, many of BFRs are persistent, lipophilic and have been shown to bioaccumulate [8,9]. They were found in the environment far away from their place of use [10–12]. Due to these facts, BFRs have become a cause for concern as potential endocrine disruptors.

Among many BFRs, there are four groups with the highest consumption; these are polybrominated biphenyl ethers, hexabromocyklododecane, polybrominated biphenyls and tetrabromobisphenol A (TBBPA) [13]. Tetrabromobisphenol A with its global consumption of 210,000 tonnes per year is a widely used BFR [7], and was therefore selected for this study. The primary use of TBBPA is as a reactive flame retardant in epoxy and polycarbonate resins that are used in the production of circuit boards. About 10% of TBBPA has additive applications in several types of polymers [14]. When used as a reactive component, TBBPA is incorporated into the structure of the polymer and is hard to release. However, when used as an additive component, TBBPA is not part of the polymer structure and can be released more easily [6]. Nevertheless, both forms have been shown to distribute TBBPA and its derivates into their surroundings [15,16].

TBBPA has been observed in several localizations in the environment. The most common sources of emission are effluents from factories producing BFRs [16]. TBBPA has also been found in river and marine sediments in Japan [17] and sewage sludge in Sweden and Canada [16,18]. It has been detected even in air and dust samples, for example in the indoor air of electronic recycling plants and in other work environments [19]. TBBPA was detected in the air and dust of two houses in Japan and it was proven that it is preferentially adsorptive to dust [20]. TBBPA was also found in the interior dust of a television cabinet [21]. It is likely that dust plays

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an important role in human exposure to TBBPA, which confirms the findings of Jakobsson et al. [22], who investigated a group of computer technicians and detected TBBPA in 8 out of 10 blood samples. TBBPA can also be transported through the placenta to the foetus and has been found in the umbilical blood of humans [23].

The effects of TBBPA on the organism could be miscellaneous. It was shown that TBBPA can bind strongly to the thyroid hormone transport protein – transthyretrin (TTR) in vitro with an even higher affinity than thyroxin [24]. The function of TTR is to transfer thyroxin and vitamin A. TBBPA was also observed to inhibit the binding of triiodthyronine to the thyroid hormone receptor and stimulate proliferation of GH3 cells (TH-dependent pituitary cell line) [25,26]. Kitamura et al. [27] reported that TBBPA disrupts amphibian metamorphosis, which is stimulated by thyroid hormone. Beside the thyroid hormone system, even the oestrogen hormone system can be affected by TBBPA. It was shown that TBBPA or its metabolites can bind to an oestrogen receptor in vitro [28] and induce proliferation of oestrogen-dependent MCF-7 cells [29,30] or Mit/E2 cells [25].

Not much is known about the effect of TBBPA on the mammalian reproduction system in vivo. The study of Van der Ven et al. [31] performed on Wistar rats confirmed that TBBPA can interact with the thyroid hormonal system in vivo. During this experiment, the levels of circulating thyroxin, one of thyroid hormone forms, were found to be decreased. TBBPA could thus negatively influence development of the offspring because the normal level of the thyroid hormone is essential for foetal and neonatal development. Rats exposed to TBBPA also displayed increased gonad weight and plasma levels of testosterone in F1 males. These observations suggest that TBBPA could affect the androgen hormonal system as well [31]. Taken together, the findings indicate that TBBPA can negatively affect the entire mammalian reproduction system. Also its structural similarity to endocrine disruptor bisphenol A, which has been shown to negatively influence the mouse reproductive system [3], suggests its similar effects. In view of these concerns, we decided to perform a multigenerational reproductive-toxicological study to assess the effect of TBBPA on the reproductive parameters of mice during long-term exposure. This study is focused on the evaluation of male gonadal pathology, sperm quality and expression of selected genes aimed to assess how TBBPA may affect the male reproductive parameters.

2. Materials and methods

2.1. Animals and treatment

For our experiment we used the CD1 outbred mice strain (An Lab Ltd., Prague, Czech Republic) with high heterozygosity and an average litter size (12–13 pups/litter). Mice (experimental and control groups) were kept under standard experimental conditions (constant temperature (23–24 °C), humidity (60 ± 5%) and 12-hour-light regime) in the breeding facility of the Institute of Molecular Genetics, v. v. i., Prague. Animals were fed by soy-free feed (Ssniff, Soest, Germany). This type of food was used to avoid any additional influence on the animals by phytooestrogen (genistein), which is present in soy. The diet and water were administered ad libitum and all stress factors were reduced to a minimum. Experimental groups were treated with a low dose of TBBPA (Fig. 1), (Sigma, Prague, Czech Republic), which was dissolved in drinking water. The concentration of TBBPA in water was 200 μg/l. The daily dose of water consumed by the mice was calculated (5 ml/day) to expose them to 1 μg of TBBPA/mouse/day, which is equivalent to 35 μg/kg. In our experiment, the following groups were evaluated: parental animals were bred to form F1 generation. In the parental generation, control animals were not exposed to TBBPA, and in the experimental group only females were exposed during gestation. In F1 generation there were two sub-groups – **C** (control group) and **T** (group exposed to TBBPA). In group T, the pups were exposed to TBBPA during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood. In group C, the pups were not exposed to TBBPA at all. F1 generation animals were bred up to the age of 70 days as follows – mother and father from group **C** formed the group **CC**; both parents from group T formed group **TT** (interbreeding), mother from group C and father from group T formed by outcrossing group **CT** and mother from group T and father from group C formed by reverse outcrossing group **TC**. Pups of F2 generation were exposed to TBBPA only in groups TC and TT (group TC was not exposed to TBBA) (Fig. 1). Animals were killed at the age of 70 days and subjected to analysis. In each group 20 animals were analyzed.

2.2. Anogenital distance, body and organ weight

Animals in the control and experimental groups were killed at the same age of 70 days. Anogenital distance (AGD) and body weight were measured and the reproductive organs were dissected and weighed. Left and right testes, epididymis, seminal vesicles and prostate were separated, cleaned and weighed individually.

2.3. Preparation of cells

Spermatozoa were obtained from proximal fifth of the left and right cauda epididymis and released to 100 μl of warmed (37 °C) PBS (phosphate-buffered saline) each for 10–15 min at 37 °C. The concentration of spermatozoa was evaluated in a haemocytometer chamber under 100× magnification. Part of the epididymal spermatozoa were taken for assessing viability, morphology and apoptotic stage. The rest of the suspension was washed twice in PBS, centrifuged for 15 min at 200 × *g* and smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on every glass slide; number of analyzed animals in each group was 20.

2.4. Sperm morphology and viability

To assess the morphological state of spermatozoa, 10 μl of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled according to the protocol using Spermac Stain System (Ferti Pro, Beernem, Belgium). Another method used for evaluation of the sperm morphological status was indirect immunofluorescence with specific monoclonal antibodies (Hs-8 and Hs-14) against intra-acrosomal proteins (see lower). Antibodies against intra-acrosomal proteins in combination with nuclei staining allow assessment of the morphology of sperm head and acrosome under the fluorescence microscope [32,33]. To determine the viability of epididymal spermatozoa a Live/Dead sperm viability kit was used (Invitrogen, Eugene, USA) according to the laboratory manual. After incubation, 10 μl of suspension was placed onto a glass slide and evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40× Nikon Plan Fluor 40/0.75.

2.5. Indirect immunofluorescence

Monoclonal antibodies against intra-acrosomal proteins (Hs-8 and Hs-14) prepared in our laboratory are routinely used to test the acrosome state [32,33]. Epididymal spermatozoa loaded on glass slides were fixed for 10 min with acetone. After rinsing with PBS, the slides were incubated overnight at 4 °C with monoclonal antibodies (diluted to an immunoglobulin concentration of 20 μg/ml). After thorough washing with PBS, the smears were incubated with anti-mouse IgM (μ-chain specific) fluorescein isothiocyanate (FITC) conjugate (Sigma, Prague, Czech Republic), diluted 1:128 in PBS and incubated for 60 min at 37 °C, washed with PBS and distilled water and mounted in Vectashield H-1200 DAPI (Vector Laboratories Inc., Burlingame, CA). The Annexin V-FITC apoptosis detection kit (Sigma, Prague, Czech Republic) was applied for detection of the sperm damage according to laboratory instructions. Samples were examined with a Nikon Eclipse E400 fluorescent microscope equipped with a Nikon Plan Apo VC 60/1.40 oil objective and photographed with a CCD 1300-VDS camera (Vosskühler GmbH, Osnabrück, Germany) with the aid of the NIS-ELEMENTS Ar imaging software (Laboratory Imaging Ltd., Prague, Czech Republic).

2.6. Histological analysis and tissue morphometry

The right testis was fixed in 4% formaldehyde in PBS. The standard paraffin-embedded 2–3 μm thick tissue sections were prepared and stained by haematoxylin–eosin staining. Tissue specimens were evaluated under a light microscope. In all specimens, 100 seminiferous tubules were analyzed by computer-assisted morphometry. The thickness of the germinal epithelium and diameter of the seminiferous tubules were measured. The results were statistically evaluated.

2.7. TUNEL analysis

The number of apoptotic cells in tissue sections of the control and experimental animals was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using an in situ detection kit (Promega, Madison, USA) according to the manufacturer's instruction. In brief, a paraffin-embedded tissue section was rehydrated in water, fixed in 4% formaldehyde, incubated in proteinase K solution (20 μg/ml) for 5 min, washed 2× in PBS, incubated for 10 min in equilibration buffer and finally exposed for 60 min to the labelling buffer containing both FITC-labelled dUTP and terminal deoxynucleotidyl transferase (control samples without terminal deoxynucleotidyl transferase or treated with DNase were also prepared). Before examination, TUNEL-labelled samples were washed in SCC (saline-sodium citrate buffer) and in water, mounted by Vectashield with DAPI dye to visualize the nuclei. Specimens were evaluated under a fluorescent microscope. In all specimens, the number of TUNEL positive cells in 20 cross-sectioned seminiferous tubules was

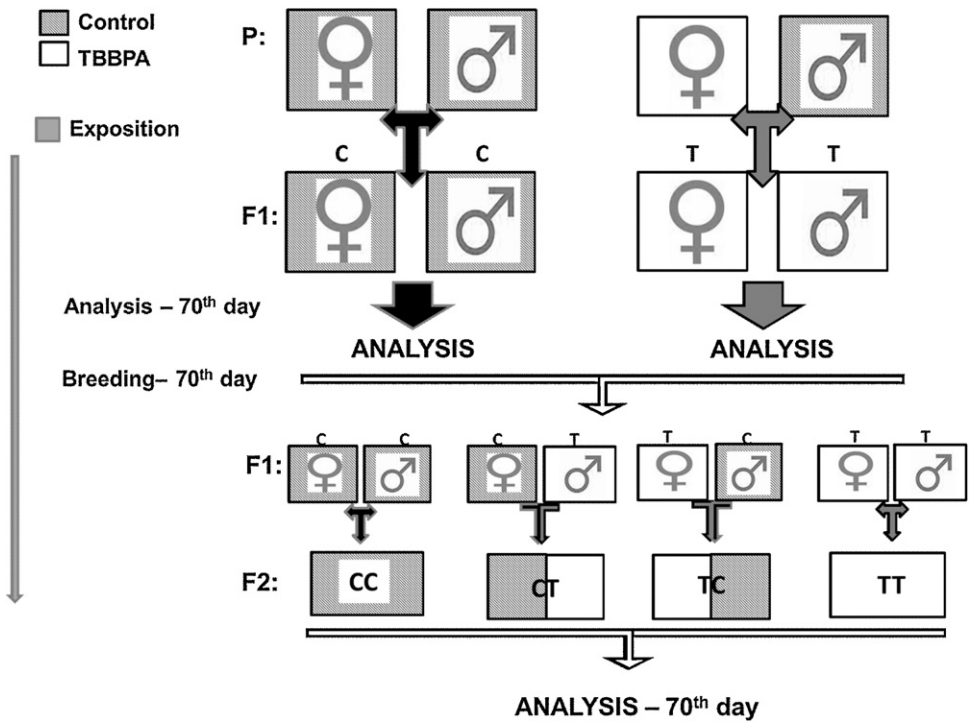

Fig. 1. Breeding diagram. Animals in parental generation were bred to form the F1 generation consisting of two groups (**C**; **T**). In group **C**, born pups were not affected by TBBPA. In group **T**, the pups were exposed to TBBPA in utero and postnatally. Animals of the F1 generation were bred to the age of 70 days as follows – mother and father from the group C formed group **CC** of the F2 generation; both parents from group T formed group **TT** (interbreeding), mother from group C and father from group T by outcrossing formed group **CT** and mother from group T and father from group C formed by reverse outcrossing group **TC** Pups born in the F2 generation were exposed to TBBPA only in groups TC and TT.

counted. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically analyzed.

2.8. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

First, total RNA was extracted from the testicular tissue. A Tri-Reagent kit (Sigma, Prague, Czech Republic) was used for RNA isolation. RNA was isolated from the left testes of CD1 mice, 1 ml of Tri Reagent was added, and the samples were then processed according to manufacturer's instructions. Isolated RNA was stored at −70 °C. The RNA quality and purity was measured spectrophotometrically in a spectrophotometer Helios α (Thermo Electron Corporation, Marietta, USA). The synthesis of cDNA was done using 5 μg of purified RNA with addition of 1 μl DNase I (Invitrogen, Carlsbad, USA), 1 μl DNase I reaction buffer (Fermentas, Burlington, Canada) and H_2O to reach a volume of 10 μl. This mixture was incubated for 30 min at 37 °C in a Touchgene Gradient Thermal Cycler (Techne, Burlington, USA). After incubation, 1 μl EDTA (Fermentas) was added and the mixture was further incubated at 65 °C for 10 min. 30 μl of the reaction mixture (8 μl of reaction buffer for M-MuLV reverse transcriptase (Fermentas, Burlington, Canada), 5 μl 10 mM 4dNTP (Fermentas, Burlington, Canada), 0.3 μl RiboLock inhibitor (Fermentas, Burlington, Canada), 1 μl oligo (dT) + random primers (Promega, Madison, USA) and 15.2 μl H_2O) was then added to the samples. The mixture was incubated for 60 min at 42 °C followed by 10 min at 70 °C and in the end was maintained at 4 °C. Obtained cDNA was stored at −20 °C. For RT-qPCR – 5× diluted cDNA was used. Used primers are summarized in Table 1. RT-qPCR reaction was carried out in PCR strips (BioRad, Prague, Czech Republic) and all work was performed in a sterile PCR box (Biosan, Riga, Latvia). For each reaction 2 μl 5× diluted cDNA, 10 μl SYBR Green Master Mix (Fermentas), 0.5 μl primer and 7 μl H_2O was used. All reactions were performed in duplets in a PCR cycler (Eppendorf, Prague, Czech Republic). The relative amount of mRNA in each sample was calculated from the measured CT values. The control was set at 100% and experimental samples were compared to the control (samples from the F1 generation were compared to the group C and samples from the F2 generation were compared to the group CC). The expression of the gene for peptidylprolyl isomerase A (PPIA) was used to normalize the measured values. The tested genes and primer sequences are summarized in Table 1. Number of analyzed animals in each group was 12 and for each group were performed 12 RT/qPCR reaction.

2.9. Statistical analysis

Statistical analysis was performed in STATISTICA 7.0 (StatSoft, Prague, Czech Republic). The number of offspring in the first generation was analyzed by the Mann–Whitney *U*-test. For analysis of the second generation the Kruskal–Wallis ANOVA test was used. Post hoc analysis in the second generation was done by using MCMR. The weight of the body and organs was compared using the analysis of variance (ANOVA), post hoc analysis was performed using the Newman–Keuls test. The weights of individual organs were compared using the analysis of covariance (ANCOVA) and the body weight was used as a covariate. Data obtained from sperm analysis and TUNEL method was analyzed using the Kruskal–Wallis ANOVA; post hoc analysis was performed again using MCMR. Differences in gene expression were analyzed by ANOVA; post hoc analysis was performed using the Newman–Keuls test. A *P*-value lower than 0.05 was identified as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Number of progeny; body and organ weight

During the developmental period male mice were exposed to TBBPA (during gestation, lactation, pre-pubertal and pubertal period, and up to adulthood). To evaluate the effect of TBBPA on the male reproductive tract the body and organ weight, number and sex ratio of progeny and anogenital distance (AGD) were measured. Individuals from group T (see Section 2.1) were compared with the control animals from group C – F1 generation. Animals from the experimental groups of the F2 generation (CT, TC, and TT) were also compared with the control animals (CC) from the F2 generation. This procedure was used in all subsequent measurements.

TBBPA has no effect on the number of progeny and sex ratio in both generations. AGD and body weight were not affected in both generations as well. In the group TT of F2 generation, significantly reduced testicular weight was observed. In this group, increased weight of the prostate and seminal vesicles was also noticed. The weight of epididymis in animals from group CT of the F2 generation was also significantly increased. All measured values are summarized in Table 2.

Table 1
Primer sequences.

Genes	Accession no.	Nucleotide sequence	Size of PCR product (bp)
Acr	NC_000081.5	5′-cacgtgtggacctcattgac-3′ 5′-gtagtcccaggtggctgtgt-3′	248
Ar	NM_013476.3	5′-ggaccatgttttacccatcg-3′ 5′-tcgtttctgctggcacatag-3′	171
Apg-1	NM_011020.3	5′-gtcagaccttcccctgaaca-3′ 5′-gctccttgactgcaggaatc-3′	215
Hsp60	NM_010477.4	5′-cttcaggggttgtcacaggt-3′ 5′-atctattgccaaggagggct-3′	137
Hsc70t	NM_013558.2	5′-cctgaccaaggaggagattg-3′ 5′-tccttcagaccctcatcacc-3′	153
Hsp70-2	NM_008301.4	5′-gcgcctcacccaactagata-3′ 5′-gatctccaccttgccatgtt-3′	145
Bax	NC_000073.5	5′-caacttcaactggggccgcg-3′ 5′-tggatccagacaagcagccgc-3′	150
Bcl-2	NC_000067.5	5′-cagggatgtcacccctggtgg-3′ 5′-aggcatcccagcctccgttatcc-3′	104
Sox9	NM_011448.4	5′-gctggaagtcggagagccgaga-3′ 5′-agagaacgaaaccggggccac-3′	147
PPIA	NC_000077.5	5′-agctctgagcactggagaga-3′ 5′-gccaggacctgtatgcttta-3′	158

Table 2
Effect of TBBPA on body and organ parameters in CD1 mice. Number of analyzed animals in each group (n) was 20.

Groups	n	AGD (cm)	Body weight (g)	Testes (mg)	Prostate (mg)	Seminal vesicles (mg)	Epididymis (mg)
C	20	1.32 ± 0.18	29.39 ± 3.11	216.65 ± 47.01	101.65 ± 23.65	194.76 ± 34.78	72.41 ± 12.15
T	20	1.26 ± 0.13	31.44 ± 2.77	224.53 ± 23.59	112.53 ± 22.74	208.93 ± 40.90	76.73 ± 9.02
CC	20	1.39 ± 0.18	31.32 ± 1.54	223.25 ± 26.86	110.88 ± 25.05	193.19 ± 36.61	70.38 ± 5.82
CT	20	1.29 ± 0.18	30.22 ± 3.58	218.27 ± 29.72	113.47 ± 30.59	204.27 ± 42.04	**73.93 ± 8.21****
TC	20	1.32 ± 0.08	30.71 ± 1.81	221.33 ± 17.94	106.00 ± 42.78	216.44 ± 17.68	72.56 ± 7.37
TT	20	1.37 ± 0.18	31.47 ± 3.88	**203.27 ± 28.24***	**127.13 ± 24.28***	**232.25 ± 33.79****	72.13 ± 7.32

Mean ± SD.
* $P<0.05$.
** $P<0.01$.

Table 3
Effect of TBBPA on sperm parameters of CD1 mice treated with TBBPA. The sperm state was analyzed by monoclonal antibodies against intra-acrosomal sperm proteins (pHs-14; pHs-8), Hoechst 33342 and Annexin V kits. The mixture of sperm suspension was smeared onto glass slides for immunocytochemical analysis. Two hundred cells (sperm) were evaluated on every glass slide; number of analyzed animals in each group (n) was 20.

Groups	n	Sperm morphology (% of normal cells)	Sperm viability (% of live cells)	Annexin V (% of positive cells)	HS-14 (% of acrosome positive cells)	HS-8 (% of acrosome positive cells)
C	20	92.88 ± 2.74	90.24 ± 3.01	51.94 ± 10.28	61.53 ± 7.65	57.18 ± 5.43
T	20	92.70 ± 5.09	89.13 ± 2.36	49.67 ± 11.08	63.13 ± 7.77	59.47 ± 7.03
CC	20	89.87 ± 2.24	89.68 ± 1.77	51.06 ± 9.01	61.87 ± 2.41	57.18 ± 1.68
CT	20	89.06 ± 2.77	89.69 ± 2.33	50.13 ± 14.15	61.81 ± 7.52	55.81 ± 3.15
TC	20	88.44 ± 3.13	89.89 ± 1.17	59.89 ± 10.56	64.56 ± 2.40	55.00 ± 3.04
TT	20	90.27 ± 1.98	90.33 ± 1.91	43.67 ± 9.80	52.60 ± 4.07	55.00 ± 3.00

Mean ± SD.

3.2. Sperm parameters

To assess the effect of TBBPA on sperm quality, the sperm morphology, viability and state of acrosome were evaluated. In all groups about 90% of morphologically normal sperm was observed, and the differences between groups were minimal. A similar pattern was observed when evaluating the sperm viability. The apoptotic stage of spermatozoa was assessed by Annexin V. In all groups, similar numbers of Annexin-positive cells were obtained by measurements. In animals from group TC of F2 generation, a slightly increased number of Annexin-positive cells was noticed, but this value did not reach significant levels. The state of the acrosome was tested with monoclonal antibodies HS-8 and HS-14. Acrosome staining was comparable in all groups of both generations. Data are summarized in Table 3.

3.3. Histology of testes

Histological analysis was performed using testis paraffin sections. Morphology of seminiferous tubules and the process of spermatogenesis were evaluated visually under the microscope. In experimental groups no pathological patterns in morphology of seminiferous tubules were observed compared to the control. Also, the process of spermatogenesis was not interrupted. For all groups in both generations, spermatogonia, spermatocytes and prolonged spermatocytes released into the lumen of seminiferous tubules were visible (Fig. 2).

However, morphometrical analysis revealed changes in other histological parameters. Epithelial thickness and tubule diameter were analyzed. Whereas the diameter of the seminiferous tubule did not show any significant changes, the seminiferous epithelium

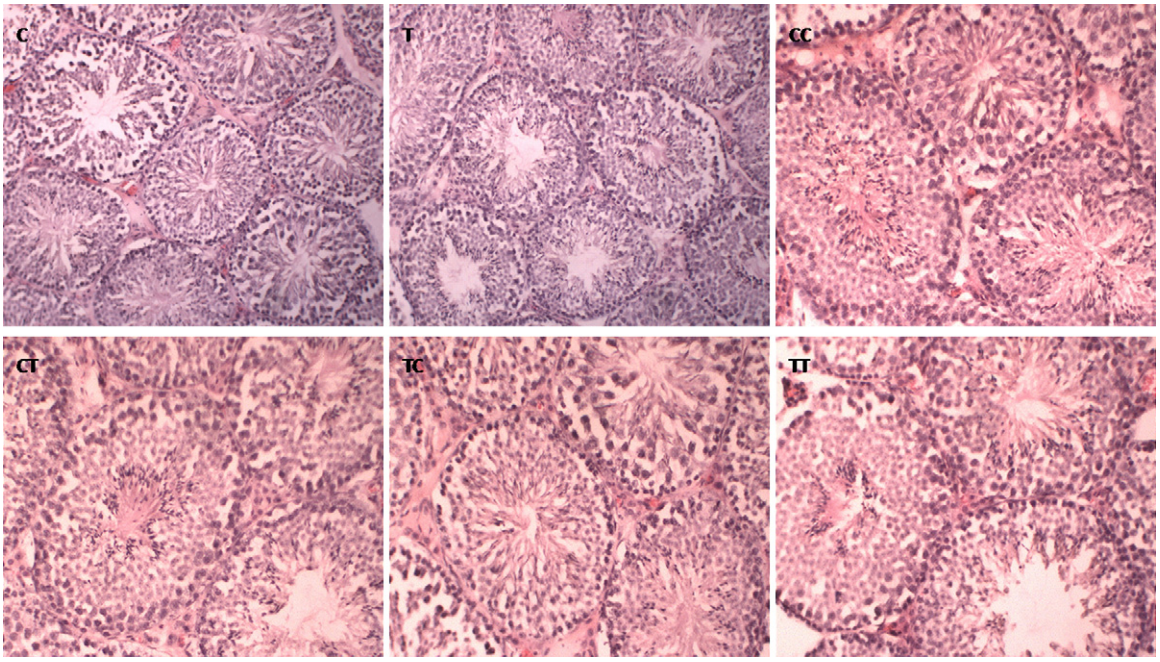

Fig. 2. Histological analysis of the testes from control and experimental animals. Paraffin sections were placed onto glass slides and stained by eosin (cytoplasm) and haematoxylin Harris (nucleus). Histological samples were analyzed under the microscope. Normal morphology and the process of spermatogenesis were observed in all experimental groups and compared to the control groups. Magnification 600×.

Table 4
Morphometrical and TUNEL analysis of seminiferous tubules. Testis paraffin sections were placed onto glass slides, where epithelial thickness as well as tubule diameters were measured and TUNEL analysis was performed. In all specimens, the thickness of the germinal epithelium and diameter of the 100 seminiferous tubules were analyzed and the number of TUNEL positive cells in 20 cross-sectioned seminiferous tubules was counted; number of analyzed animals in each group (n) was 20.

Groups	n	Epithelial thickness (μm)	Tubule diameter (μm)	Number of TUNEL positive cells
C	20	61.8 ± 3.61	225.5 ± 12.3	25.65 ± 1.62
T	20	**57.3 ± 4.61***	214.4 ± 11.5	**42.27 ± 6.10****
CC	20	59.5 ± 3.05	215.6 ± 17.4	24.67 ± 1.37
CT	20	58.0 ± 3.56	220.1 ± 23.7	26.00 ± 3.22
TC	20	**56.1 ± 3.13***	219.7 ± 23.44	**31.00 ± 2.10****
TT	20	**56.4 ± 3.22***	218.1 ± 21.3	**30.50 ± 2.81****

Mean ± SD.
* $P < 0.05$.
** $P < 0.01$.

was significantly lower in groups T (F1 generation) and TC; TT (F2 generation). Results are summarized in Table 4.

3.4. TUNEL analysis

To determine the number of apoptotic cells in the testes the TUNEL analysis of testis paraffin sections was performed. The highest increase of apoptotic cells in the testes was observed in group T of the F1 generation, where the number of apoptotic cells was almost two times higher in comparison to the control group. The increased number of apoptotic cells was also observed in groups TT and TC of the F2 generation, where about 25% more TUNEL-positive cells was measured compared to the control group (Table 4).

3.5. Real time PCR of testicular genes

Genetic analysis was performed by the quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) with primers for the genes of acrosomal proteins, androgen-responsive genes, heat shock protein (Hsp) genes, genes encoding proteins responsible for the regulation of apoptosis and a Sertoli cell-specific gene. The acrosome-specific gene tested was proacrosin (Acr). The androgen receptor (Ar) gene was selected as a member of the androgen-responsive genes. Genes tested for heat shock proteins were Hsp70-2, Hsc70t, Hsp60, and APG-1. The genes selected for their relation to apoptosis were Bcl-2 and Bax and the Sertoli cell-specific gene tested was Sox9 (Table 5). The Acr gene expression was comparable in all groups in both generations. No significant changes of gene expression for the androgen receptor were observed among the males of F1 generation. Contrary to that, the groups TC and TT had significantly reduced ($P < 0.001$) expression of this gene by about 20% compared with the control.

Our experiments showed relatively large changes in the expression of selected heat shock protein-encoding genes, with the exception of heat shock protein APG-1. The level of gene expression of APG-1 did not show any significant changes between the experimental and control groups. In contrast, a significantly reduced expression of the gene for Hsp70-2 in both generations ($P < 0.001$) was observed. The most reduced expression of this gene was observed in groups T of the F1 generation and TT of the F2 generation, reaching half the values compared with the control groups. A comparable pattern was observed for the Hsp60 gene, where the expression was significantly reduced in all experimental groups, both in F1 and in F2 generations ($P < 0.001$). Reduced expression was most apparent in the group T of the F1 generation and groups TC and TT of the F2 generation, where it reached nearly half the

Table 5
Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) analysis of testicular acrosome-, androgen-, heat shock- and apoptosis-related genes. In this study, genes for proacrosin (Acr), androgen receptor (Ar), heat shock proteins – Apg-1, Hsp70-2, Hsp60 and Hsc70t, apoptosis-related genes such as Bax and Bcl-2 and Sertoli cell-specific gene Sox9 were tested. The table illustrates relative expression of the selected genes. Control groups represent 100% and the percentage in the experimental groups represents the ratio of gene expression between the treated groups (T, CT, TC, TT) and the control groups (C, CC). Number of analyzed animals in each group was 12 (*nb*) and for each group were performed 12 RT/qPCR reaction (*nt*).

Groups	*nb*/*nt*	Acr (%)	Ar (%)	APG-1 (%)	Hsp70-2 (%)	Hsp60 (%)	Hsc70t (%)	Bax (%)	Bcl-2 (%)	Sox9 (%)
C	12/12	100.0 ± 11.3	100.0 ± 6.6	100.0 ± 7.2	100.0 ± 8.11	100.0 ± 8.2	100.0 ± 12.6	100.0 ± 11.5	100.0 ± 12.2	100.0 ± 10.6
T	12/12	96.3 ± 12.5	110.0 ± 6.2	85.3 ± 12.6	**54.2 ± 7.4****	**60.7 ± 6.9****	**142.7 ± 13.5****	**144.7 ± 10.6****	**60.3 ± 9.1****	**45.5 ± 7.5****
CC	12/12	100.0 ± 11.5	100.0 ± 6.4	100.0 ± 7.9	100.0 ± 9.8	100.0 ± 11.2	100.0 ± 12.4	100.0 ± 11.5	100.0 ± 11.1	100.0 ± 20.3
CT	12/12	96.3 ± 11.7	94.8 ± 5.1	107.8 ± 13.9	**79.2 ± 8.4****	**72 ± 6.4****	102.0 ± 14.2	108.5 ± 13.3	91.0 ± 12.2	93.1 ± 15.11
TC	12/12	**108.8 ± 11.7**	**74.3 ± 7.9****	**116.8 ± 10.3**	**59.7 ± 8.9****	**59.8 ± 10.8****	**140.5 ± 7.6****	**128.8 ± 8.1****	**82.5 ± 9.1***	**53.6 ± 13.1****
TT	12/12	89.3 ± 10.2	**85.7 ± 6.9****	91.5 ± 6.4	**51.5 ± 8.1****	**56.6 ± 10.8****	**145.8 ± 14.8****	**134.8 ± 9.5****	**71.8 ± 9.4****	**38.6 ± 3.2****

Mean ± SD.
* $P < 0.05$.
** $P < 0.01$.

values compared with the control group. The expression level for Hsc70t was higher in group T of the F1 generation and groups TC, TT of the F2 generation ($P < 0.001$). The highest increase in expression was observed in groups T (F1 generation) and TT (F2 generation), where they reached almost 50% higher values than they did in the control group.

A differential expression pattern was also observed in genes encoding apoptotic genes Bax and Bcl-2. The most increased gene expression of Bax was observed in group T (F1 generation) and groups TC, TT (F2 generation), where 40% and 30% higher expression compared with the control group was detected. In contrast, low levels of expression were observed in the case of anti-apoptotic gene Bcl-2. Significantly reduced expression was detected in group T (F1 generation) ($P < 0.001$) and groups TC ($P < 0.05$), TT ($P < 0.001$) (F2 generation). The highest decrease in gene expression was observed in group T of the F1 generation, where the expression level was reduced by 40% compared with the control group.

The expression of Sertoli cell-specific gene Sox 9 was significantly decreased in the experimental groups, particularly in group T of F1 generation ($P < 0.001$) and groups TC and TT of F2 generation ($P < 0.001$) (Table 5).

4. Discussion

In this study we tested the effect of TBBPA on the reproduction parameters of CD1 mice. We nalyzed the following parameters: the numbers of progeny, body and organ weight, anogenital distance, sperm quality, histopathology of testes, apoptotic stage of testicular cells and activity of selected genes (acrosomal, androgen-responsive, heat shock and apoptotic genes). For our experiment, we used the CD1 outbred mice strain, because it simulates the human population more closely than inbred strains given its high heterozygosity. Experimental mice were exposed to the long-term effects of TBBPA. The substance was administered dissolved in water at a concentration of 200 μg/l.

Previous studies have shown that TBBPA can affect the thyroid hormonal system, mainly by binding to thyroid hormone transport protein – transthyretin [24–26]. Also the oestrogenic hormone system can be potentially affected by TBBPA. It was demonstrated that TBBPA is capable of binding the oestrogen receptor in vitro [28]. TBBPA can also induce apoptosis of TM4 Sertoli cells in vitro [34], but little is known about its effect on the reproductive parameters in vivo.

Our histological analysis showed no visible abnormalities or pathological changes in the morphology of seminiferous tubules of experimental animals (Fig. 1.). However, TUNEL analysis of histological sections of the testes showed a significantly increased number of apoptotic cells in the testes of experimental animals in groups T (F1 generation) and TC, TT (F2 generation) (Table 4). Mice in these groups also displayed significantly increased expression of the gene for pro-apoptotic protein Bax and decreased expression of the gene for anti-apoptotic protein Bcl-2 (Table 5). Proteins Bax and Bcl-2 play a key role in the activation of cell apoptosis. Anti-apototic protein Bcl-2 is located on the outer mitochondrial membrane and binds to the pro-apoptotic protein Bax. If this bond is not present, protein Bax becomes active and forms a homo-oligomeric channel, allowing an influx of ions into the mitochondria and thus initiating the activation of the apoptotic process. Therefore, unbalanced expression of these proteins could lead to the induction of apoptosis [35].

The groups in which an increased number of apoptotic cells was detected also had a significantly decreased expression of the Hsp60 gene (Table 5). Hsp60 is a mitochondrial protein that serves as a chaperone of the newly imported proteins and also helps transport proteins into the mitochondrial matrix and intra-membrane

space. The Hsp60 function is essential for the proper functioning of mitochondria [36,37].

Morphometrical analysis revealed significantly thinner seminiferous epithelium in groups T (F1 generation) and TC, TT (F2 generation) (Table 5). This may be a result of an increased incidence of apoptosis in the testis of mice in these groups. According to the study of Ogunbayo et al. [34], who showed that TBBPA is able to induce apoptosis in Sertoli cells, we assume that the apoptosis of Sertoli cells is the main cause of the decrease of thickness of the seminiferous epithelium. To support this hypothesis of TBBPA affecting Sertoli cells, we analyzed the expression of the nuclear transcription factor Sox9 gene. Sox9 is preferentially expressed in Sertoli cells and can serve as a marker of Sertoli cells. We detected decreased expression of the Sox 9 gene in groups T (F1 generation) and TC; TT (F2 generation), the same groups in which we detected the increased incidence of apoptosis in the testes.

Based on our results we can assume that the increase of apoptosis in the testes of groups T (F1 generation) and TC, TT (F2 generation) was caused by damage or malfunction of the mitochondria. This hypothesis is supported by the results of Ogunbayo et al. [34], who demonstrated that TBBPA causes dramatic changes in the mitochondrial membrane potential and subsequently the apoptosis of TM4 Sertoli cells in vitro. It is likely that changes in the mitochondrial membrane potential induce the release of various pro-apoptotic factors and subsequently induce programmed cell death in these cells in vitro [38].

In all experimental groups of F1 and F2 generations we observed decreased expression of the gene for Hsp70-2 (Table 5). Hsp70-2 is expressed during the meiotic phase of spermatogenesis, mainly in the pachytene spermatocytes [39]. It has been shown that the lack of expression of this gene causes disruption of spermatogenesis and induces apoptosis of spermatogenic meiotic cells [40]. Widlack et al. [41] showed that constitutive expression of active HSF1 leads to the induction of caspase-3-dependent apoptosis, and among others, down-regulation of Hsp70-2. HSF1 is the primary transcription factor responsible for the response to various forms of cellular stress and its constitutive expression in cells simulates exposure to permanent stress [42]. This indicates that TBBPA could cause stress conditions in spermatogenic cells that could lead to a decrease in the expression of Hsp70-2, which in turn could contribute to an increased number of apoptotic cells in the testes of experimental animals.

We also detected the expression of Hsc70t gene. Hsc70t is expressed specifically in the testes during the post-meiotic stages of spermatogenesis with the highest incidence in elongating spermatids [43]. It has been shown that the lack of this protein causes rapid loss of sperm motility [44], but its exact function is not yet known. In groups T (F1 generation) and TC, TT (F2 generation) we observed increased expression of this gene (Table 5). This again could be induced by stress, as a result of the exposure of experimental animals to TBBPA.

Another gene detected was proacrosin (Acr). Proacrosin is one of the main acrosomal proteins that becomes active during the acrosome reaction and binds to the glycoproteins of zona pellucida [45]. This gene was selected as a marker of acrosomal damage. The expression of this gene was comparable in all groups in both generations (Table 5). Data obtained from immunocytochemistry using specific antibodies against intra-acrosomal proteins (Hs-14 and Hs-8) did not reveal any damage to the acrosome (Table 3). It can therefore be assumed that TBBPA has no negative influence on the development and function of the sperm acrosome.

The expression of the gene for androgen receptor (Ar) in groups TC and TT (F2 generation) was reduced (Table 5). Ar is a nuclear receptor that becomes active after the binding of its ligand (natural ligands – testosterone and dihydrotestosterone). After its activation Ar is translocated to the nucleus where it functions as a transcriptional factor and activates transcription of androgen-dependent genes. The reduced expression of the Ar gene was also observed in CD1 mice affected by anti-androgen vinclozoline [2] or in rats affected by flutamide (anti-androgen drug administered to treat cancer) [46]. It can therefore be assumed that TBBPA could have a weak anti-androgen effect, which was apparent in the second generation of the experimental animals.

The groups most affected by TBBPA were T (F1 generation) and TT (F2 generation). In the second generation, the influence of TBBPA had a stronger effect than in the first generation. The reproductive organs of group TT displayed lower weight compared to the control and also decreased expression of the gene for androgen receptor up to the second generation.

Differing expression of the selected genes and increased apoptosis in the testes was detected in group TC (F2 generation). However, this group was affected less than group TT (F2 generation). We may thus assume that for the TBBPA activity it is important whether the parents were affected or not. This hypothesis is supported by the finding that changes in gene expression were observed even in group CT (F2 generation), where only the father was exposed to TBBPA (neither mother nor offspring were exposed to TBBPA). It thus seems that certain effects of TBBPA can be transmitted to the next generation. One possible route of such transfer may be epigenetic changes. It is hypothesized that exposure to environmental pollutants may induce epigenetic changes. These changes do not involve changes in the DNA sequence but may cause changes in gene expression and be transmitted to the next generations. This hypothesis was strongly supported by the study of Stouder and Paolini-Giacobino [47], who showed that endocrine disruptor vinclozoline is able to induce epigenetic changes. In their study administration of vinclozoline to pregnant female mice induced alterations in the imprinting of five paternally/maternally imprinted genes in the sperm of the offspring. Another study showed an association between imprinting errors and male infertility, specifically azoospermia [48]. Association between methylation defects and infertility itself was suggested in other studies as well [49–51]. These findings indicate that the possible mechanism of effect of environmental pollutants and the mechanism by which this effect is transmitted to the next generations could be via epigenetic changes. However, to be confirmed, this hypothesis needs further investigation.

5. Conclusion

In summary, our results provide evidence that TBBPA is capable of inducing apoptosis of testicular cells and changes in the morphometry of seminiferous tubules in CD1 mice. We assume that this involves also Sertoli cells, which provide support to developing spermatocytes. In this study, the investigated sperm parameters did not reveal any sperm damage. However, analysis of gene expression revealed changes in the expression of selected testicular genes. These genes were selected because of their essential role during spermatogenesis and because their impaired expression may negatively influence the course of this process. This two-generational in vivo study also suggests that permanent exposure to TBBPA slightly enhances its effect in the next generation depending on whether the parents were affected or not.

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SUPPLEMENT 6

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The effect of tetrabromobisphenol A on protamine content and DNA integrity in mouse sperm

Running Title: TBBA, sperm protamines and DNA integrity

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ABSTRACT:

Tetrabromobisphenol (TBBPA) is a widely used brominated flame retardant (BFR) of increasing concern to human health because of its action as an endocrine disruptor. We have previously demonstrated that TBBPA is able to increase apoptosis of testicular cells and other changes in the first and second generations of mice exposed to TBBPA. However, the potential effects of TBBPA on mouse epididymal spermatozoa have not yet been investigated. Therefore, we initiated the present study to determine whether TBBPA exposure could also result in increased DNA fragmentation in epididymal spermatozoa and whether it had an effect on the protamines as the major nuclear proteins. C57Bl/6J mouse pups (n=10) were exposed to TBBPA (experimental group) during the gestation, lactation, pre-pubertal and pubertal periods up to the age of 70 days as previously described and compared to control mouse pups (n= 10) that were not exposed. The results demonstrate that TBBPA treatment results in a significantly decreased P1/P2 ratio (0.362 vs. 0.494; $P< 0.001$), increased total protamine/DNA ratio (0.517 vs. 0.324; $P< 0.001$) and increased number of TUNEL positive spermatozoa (39.5% vs. 21.2%; $P<0.05$) observed between TBBPA and control mice, respectively. These findings indicate that TBBPA exposure, in addition to the resulting increased sperm DNA damage, also has the potential to alter the epigenetic marking of sperm chromatin through generation of an anomalous content and distribution of protamines. The possibility is now open to study whether the detected altered protamine content and DNA integrity are related to the previously observed second-generation effects upon TBBPA exposure.

Keywords: Spermatozoa, Protamines, Tetrabromobisphenol A, TUNEL assay

INTRODUCTION

Tetrabromobisphenol A (TBBPA) is a brominated flame retardant (BFR) with a global consumption of 210,000 tonnes per year (European Food Safety Authority, 2011) and is released into the environment from existing or wasted products exposing the human population. For example, TBBPA was found in the blood serum of computer technicians, in breast milk and blood serum of mothers, and has also been shown to be transported through the placenta to the foetus (de Wit et al., 2002; Jakobsson et al., 2002; Cariou et al., 2008; Kawashiro et al., 2008). BFRs are classified as endocrine disruptors because they mimic the function of endogenous hormones (Korner et al., 1998; Meerts et al., 2000; Samuelsen et al., 2001; Kitamura et al., 2002; Uhnakova et al., 2011). Few studies have examined the effect of TBBPA on the reproductive system in vivo. Experiments performed on Wistar rats (Van der Ven et al., 2008) reported a reduction of thyroxin in circulation and an increase in gonad weight and plasma levels of testosterone in F1 males. A previous study conducted in our laboratory tested the effect of TBBPA on reproductive parameters in a two-generational in vivo study using CD1 mice. We observed increased apoptosis of testicular cells and changes in the expression of genes playing important roles during spermatogenesis. A parental effect related to TBBPA in the second generation was also found in this study (Zatecka et al., 2013).

The present study was designed to examine the effect of TBBPA on spermatozoa as the carriers of parental genetic information. The spermatozoon is a specialized cell with a condensed nucleus in which the majority of the DNA is tightly packaged in toroidal structures by protamines (Oliva & Dixon 1991; Oliva 2006; Balhorn 2007; Carrell et al., 2008; Oliva et al., 2011). In mice as well as humans there are two types of protamines: protamine 1 (P1) and protamine 2 (P2). Several studies have reported an altered amount of protamines in the sperm cell of infertile patients and a correlation with DNA fragmentation (Corzett et al., 2002; Oliva 2006; Balhorn 2007; Carrell et al., 2008; de Mateo et al., 2009). Furthermore, haploinsufficiency of only one of the protamine 1 gene (Prm1) or protamine 2 gene (Prm2) alleles in knockout mice models results in severely altered spermatogenesis, increased DNA damage and sperm-cell apoptosis (Cho et al., 2001; 2003). Indeed, one of the main functions of sperm protamines is related to the protection of the paternal genetic message through making it inaccessible to nucleases or mutagens (Oliva 2006). This is especially relevant as sperm genome integrity seems to be necessary in order to accomplish fertilization (Tomsu et al., 2002; Simon et al., 2011). However, protamines may also be involved in paternal genome imprinting during spermatogenesis and in the constitution of epigenetic marks potentially

transmitted to the oocyte upon fertilization (Oliva & Dixon 1991; Oliva 2006; Carrell et al., 2008; Hammoud et al., 2011; Castillo et al., 2014). Despite all the above studies correlating TBBPA with male reproductive effects and the links between altered protamination, DNA fragmentation and male infertility, to our knowledge there are no studies measuring the effect of TBBPA on protamination of the sperm cell and DNA integrity. Therefore, this study was performed to determine the effect of TBBPA on protamination levels and sperm DNA integrity as both parameters are correlated with male fertility.

MATERIALS AND METHODS

Animals and treatment

For our experiment we used a C57Bl/6J inbred mouse strain (An Lab, Prague, Czech Republic). Mice were kept under standard experimental conditions with constant temperature (23 - 24 °C) and a 12-hour light regime in the animal facility of the Institute of Molecular Genetics of the AS CR, v. v. i., Prague. Animals were fed on soy-free feed (LASvendi, Soest, Germany). Food and water were administered ad libitum. In this in vivo experiment there were two groups; one group exposed to TBBPA (experimental group) and the control group, both exposed in the indicated animal facility in Prague. In each group, 10 animals were analysed. Experimental animals were treated with TBBPA (Sigma, Prague, Czech Republic), which was dissolved in drinking water in a concentration of 200 μg/l. The animals were exposed approximately to 1 μg of TBBPA/mouse/day (equivalent to 35 μg/kg). The concentration of TBBPA in the environment is highly variable, but we tried to select our concentration to mimic potential in vivo exposures (Sellstrom and Jansson, 1995; Takigami et al., 2009), and also because it had been used in our previous in vivo study where we showed that TBBPA is able to influence processes occurring in the testicular tissue (Zatecka et al.,2013).

Animals in the experimental group were exposed from the day of conception (their parents were non-exposed animals) throughout the gestation, lactation, pre-pubertal and pubertal periods up to the age of 70 days, and then they were sacrificed and subjected to analysis. Each group (experimental and control) consisted of 10 individuals. The animals were exposed during all their life since conception in order to simulate real exposures of humans to environmental pollutants throughout life. All procedures were approved by the Committee for Animal Welfare and Protection.

Sperm extraction and purification

The epididymides were dissected and cleaned from any additional tissue. The caudal regions of the epididymides were separated, placed into warmed (37°C) PBS, and then they were carefully cut into small pieces, and the spermatozoa were left to release spontaneously from the epididymis in a CO_2 incubator (CO_2 5%) for 15 min. After incubation the mixture was poured through a 30 μm filter to obtain only the sperm cell fraction (Partec, Görlitz, Germany) and PBS was added to 1 ml final volume. Part of the epididymal spermatozoa was

used for assessing sperm morphology and DNA damage. The rest of the suspension was washed in PBS, centrifuged at 1240 g for 10 min at 4°C and the pellet was used for the protamine extraction.

Sperm morphology

For evaluation of the morphological state of spermatozoa, 10 µl of sperm cell suspension was placed onto a glass slide, fixed at 37 °C and labelled by Spermac Stain System according to the manufacturer's protocol (Ferti Pro, Beernem, Belgium). A total of 200 cells from each sample were evaluated as described (Elzeinova et al., 2008).

Testosterone and T3 level measurements

Blood samples (0.5 ml) were collected from males at the age of 70 days. Serum testosterone and total triiodothyronine (T3) were measured by the commercially available RIA kits (Beckman coulter, Prague, Czech Republic) according to manufacturer's instructions. Serum samples were evaluated using a gamma counter (Cobra II, Canberra Packard, USA) set for 125 iodine.

Sperm DNA damage

The level of DNA damage of epididymal sperm was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using a detection kit (Promega, Madison, USA) according to the manufacturer's instructions. The fresh sperm suspension (final concentration of $1x10^6$ cells/ml) was dropped onto 3-well diagnostic microscope slides (ThermoScientific, Portsmouth, USA) and air dried. For each well 10 µl of sperm suspension was used. Subsequently, the samples were processed according to manufacturer's instructions. Finally, the samples were mounted in Vectashield with DAPI dye (Vector, Burlingame, USA) for DNA visualization (Domínguez-Fandos et al., 2007). All specimens were evaluated with a fluorescent microscope Nikon Eclipse 400 (Nikon, Japan) equipped with a Nikon Plan Apo VC 60/1.40 oil objective (Nikon). The images were photographed with a CCD camera 1300-VDS (Vosskühler, Osnabrück, Germany) with the aid of the NIS-Elements Ar imaging software (Laboratory Imaging, Prague, Czech Republic). Two hundred cells were evaluated in each sample.

Extraction of sperm nuclear proteins

For protamine extraction 5 x 10^6 of mouse epididymal sperm cells were used. Sperm cells were resuspended in 200 µl PBS, centrifuged at 8940 g for 5 min at 4°C and the pellet was resuspended in 100 µl of 0.5% Triton X-100, 20 mM Tris, 2 mM $MgCl_2$ solution and centrifuged at 8940 g for 5 min at 4°C. After centrifugation the samples were treated as previously described (de Yebra & Oliva 1993), except that instead of using iodoacetate treatment we performed a treatment with 0.8% vinylpyridine (Sigma) for 30 min at 37°C to further inhibit formation of cysteine disulfide bonds. Finally, each sample was resuspended in 10 µl of sample buffer containing 5.5 M urea, 20% β-mercaptoethanol and 5% acetic acid. The DNA remaining in the pellet after the extraction of the nuclear proteins with 0.5 M HCl was extracted and quantified after 0.5 N perchloric acid hydrolysis (90°C, 20 min) and absorbance determination at 260 nm, measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, Delaware), as previously described (Castillo et al., 2011).

Separation and analysis of sperm nuclear proteins

Basic nuclear proteins were analysed using acid-urea polyacrylamide gel containing 2.5 M urea, 12.5 mM thiourea, 0.9 M acetic acid, 15% acrylamide, 0.1% bis-acrylamide and 0.12% H_2O_2. After polymerization, 2 µl of each sample was loaded and the gel was electrophoresed in 0.9 M acetic acid buffer for 90 minutes at 110 V (Ausio 1992). Different known quantities of a human protamine standard from a pool of human normozoospermic sperm samples (0.435, 0.87, 1.74 and 2.61 µg) were added in each acid urea electrophoretic gel (Mengual et al., 2003).

The gels were stained with EzBlue™ staining reagent (Sigma) following the manufacturer's instructions. The stained gels were scanned and the intensity of the bands corresponding to P1 and P2 was quantified with Quantity One software (Bio-Rad Laboratories, Hercules, California, USA). The data obtained from Quantity One software were used for calculating the P1/P2 ratio. A standard curve was obtained from the different concentrations of human protamine standard in order to calculate the total amount of protamines (P1 + P2) in each sample, and the P1/P2, P1+P2/DNA, P1/DNA and P2/DNA ratios were calculated (Mengual et al., 2003). A representative example of one of the acid-urea polyacrylamide gels stained by EzBlue is shown in Figure 1.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The whole RNA was extracted from the whole testicular tissue using the Tri-Reagent kit (Sigma). Briefly, to each testicle, 1 ml of Tri-Reagent was added and the tissue was then homogenized in homogenizer Precellys 24 (Bertin Technologies, Aix-en-Provence, France). Subsequently, the samples were processed according to the manufacturer's instructions. The quality and purity of the isolated RNA was measured with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Washington, USA). The cDNA was synthesized by combining 5 µg of purified RNA, 1 µl DNase I (Invitrogen, Carlsbad, USA), 1 µl DNase I reaction buffer (Fermentas, Burlington, Canada) and H_2O to reach a volume of 10 µl. This mixture was incubated for 30 minutes at 37 °C in a Touchgene Gradient Thermal Cycler, (Techne, Burlington, USA). After incubation, 1 µl EDTA (Fermentas) was added and the mixture was incubated at 65 °C for 10 minutes. The volume of 30 µl of the reaction mixture (8 µl of reaction buffer for M-MuLV reverse trancriptase (Fermentas), 5 µl 10 mM 4 dNTP (Fermentas), 0.3 µl RiboLock inhibitor (Fermentas), 1 µl oligo (dT) + random primers (Promega, Madison, USA) and 15.2 µl H_2O) was added to the samples. The mixture was incubated for 60 minutes at 42°C and then for 10 minutes at 70°C. The obtained cDNA was stored at -20°C for subsequent analysis.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed with primers for protamine 1 gene (Prm1), protamine 2 gene (Prm2), transition protein 1 gene (Tnp1) and transition protein 2 gene (Tnp2). The primer sequences are given in Table 1. For each reaction 2 µl of 5x diluted cDNA, 10 µl SYBR Green Master Mix (Fermentas), 0.5 µl primer and 7 µl H_2O were used. All reactions were performed in duplicates in a PCR cycler (Bio-Rad Laboratories). The relative amount of mRNA in each sample was calculated from the measured CT values. The control was set at 100 % and the experimental samples were compared to the control. The expression of the gene for β-actin (Actb) was used to optimize the measured values as described (Livak & Schmittgen 2001).

Statistical analysis

Experimental data were analysed using STATISTICA 6.0 and GraphPad Prism 5.04. The differences between individual parameters in the control and the TBBPA group were tested by Mann-Whitney U test. The correlations between individual parameters were expressed as Pearson product-moment correlation coefficient (r). Individual r coefficients were tested for

their significance. A p value equal or lower than 0.05 was considered to be significant (*P value ≤0.05, **P ≤0.01 and ***P ≤0.001).

RESULTS

General reproductive system measurements and animal body weight

TBBPA treatment did not result in statistically significant changes in any of the general reproductive system parameters measured (supplementary table). Recorded parameters were the ano-genital distance (AGD), weight of the reproductive organs, sperm morphology and concentration. Also, the animal body weight was not changed as a result of the treatment.

Testoterone and T3 levels

Testosterone and T3 levels were measured to determine whether the previous reported effects of TBBPA treatment in rats (Van der Ven et al., 2008) could also be reproduced in mice. As expected, TBBPA-treated animal had lower testosterone levels as compared to the control group (0.268 ng/ml±0.23 (SD) vs. 0.48 ng/ml±0.222 (SD); $P<0.05$). The average T3 levels were also lower in the TBBPA-treated animals, although in this case no significant differences were reached (1.187 nmol/l±0.324 (SD) vs. 1.301 nmol/l±0.135 (SD).

Protamine ratio and protamine content

After protein staining and visualization we measured the optical density of the bands that corresponded to P1 and P2, respectively (Fig. 1A), and the P1 to P2 ratio (P1/P2) was calculated for each sample (Fig. 1B). In animals exposed to TBBPA the P1/P2 ratio was 0.362±0.024, while in control animals the ratio was 0.494±0.052 ($P<0.001$), consistently with the normal ratio described for mice (Corzett et al., 2002). Thus, in the experimental animals the P1/P2 ratio was significantly lower as compared to the control group (Fig. 1B). Using protamine standards, we also calculated the whole protamine content in each sample. Finally, we also measured the DNA content of the samples, which allowed calculating the total protamine to DNA ratio (P1+P2/DNA) and the P1/DNA and P2/DNA ratios. In the control animals the P1+P2/DNA ratio was 0.324±0.081, while in the experimental animals we obtained a significantly higher P1+P2/DNA ratio of 0.517±0.073 ($P<0.001$; Fig. 1C). This increase in the total protamine content is due to a significant increase in the P2/DNA ratio (Fig 1E),

Sperm morphology and DNA damage

The cauda epididymal sperms obtained from control and experimental animals were subjected to morphological analysis and evaluation of DNA damage through the TUNEL assay, which

enables detection of DNA fragmentation. There were no differences in the number of sperm cells/ml recovered (the sperm cells $*10^6$/ml ± standard deviation was 17.60±6.40 in control vs 15.76±5.80 in treated animals). During the morphological analysis we evaluated the morphology of sperm heads. We did not observe any differences in the number of morphologically abnormal spermatozoa in TBBPA-treated animals compared to the control (% of average abnormal sperm ± standard deviation was 6.10±2.60 vs 7.6±2.59 respectively). However, as shown in Figure 2, we detected a significantly higher number of TUNEL-positive cells in sperms from TBBPA-treated animals (39.5±4.5% cells) as compared to the controls (21.2±3.1% cells; $P<0.05$). Moreover, the statistical analysis revealed a negative correlation between the DNA damage and the P1/P2 ratio (Fig. 3; $P<0.05$). However, we did not find any correlation between total protamine/DNA; P1/DNA; P2/DNA and sperm DNA damage. In case of P2/DNA with TUNEL we could see a trend (r=0,38) however the p value did not reach level of significance (P=0,11). We also searched for potential correlations between the sperm concentration or morphology and sperm protamination, but we did not find any significant results. We did not detect any correlation either between sperm morphology assessed using optical microscopy and protamine content. However, it will be interesting to explore in the future, using higher resolution methods, whether the detected alterations in protamine content are linked to ultrastructural defects.

Expression analysis of testicular RNA

After testes dissection we extracted whole testicular RNA and evaluated the expression of several testicular genes. Genetic analysis was performed by qRT-PCR with primers for the protamine 1 (Prm1); protamine 2 (Prm2); transition protein 1 (Tnp1) and transition protein 2 (Tnp2) genes (Table 1). The qRT-PCR method did not reveal any significant changes in the expression profile of any of the tested genes in the experimental animals (Table 2). We also calculated the Prm1/Prm2 mRNA ratio. In experimental animals, the ratio was slightly lower (average 0.56 ± 0.10 vs. 0.60 ± 0.18); however, the differences were not statistically significant.

DISCUSSION

In this manuscript we report that TBBPA exposure leads to a decreased P1/P2 ratio, increased total protamine/DNA ratio, and in an increased number of TUNEL positive spermatozoa (Figs. 1 and 2). In humans, alterations in the P1/P2 ratio have been reported in infertile patients and are associated with increased sperm DNA damage and failed assisted reproduction outcomes (Mengual et al., 2003; Aoki et al., 2005; Balhorn 2007; de Mateo et al., 2009; Castillo et al., 2011; Simon et al., 2011). According to the DNA protection hypothesis for protamines, the prediction would be that the detected increase in DNA damage would correlate with decreased total P1+P2/DNA ratios (Aoki et al., 2005; Oliva, 2006). However, our results obtained in the present work in the mouse model instead indicate a significant increase in the total P1+P2/DNA ratio in the TBBPA-treated animals, with increased TUNEL-positive sperm (Figs. 1 and 2). But not all published data fits well the DNA protection hypothesis. For example, a significantly increased P1+P2/DNA ratio was detected in the sperm cells from native semen infertile patient samples having higher DNA fragmentation levels as compared to density gradient-selected sperm with lower levels of DNA damage and lower P1+P2/DNA ratios (Castillo et al., 2011). Also, a lack of detection of significant changes in the total protamine content was reported in oligozoospermic patients as compared to controls, despite the presence of significantly different P1/P2 ratios (Mengual et al., 2003). Differently to the above data on protamine content and DNA damage, low P1/P2 ratios have been consistently associated with higher sperm DNA fragmentation indices in different studies (Aoki et al., 2005; Oliva, 2006; Figure 3). Thus, the P1/P2 and protamine/DNA (or protamine/sperm) ratios are not equivalent. Therefore, our results along with some of these previous observations suggest that the detected associations between decreased P1/P2 ratios, changes in the protamine/DNA ratios found in some studies, and increased DNA damage cannot be simply explained by the DNA protection hypothesis of protamines, but that the link is more complex.

Another hypothesis for the function of protamines is that they are involved in the imprinting of sperm chromatin (Oliva & Dixon 1991; Oliva 2006; Arpanahi et al., 2009; Hammoud et al., 2009; Castillo et al., 2014). This latter imprinting hypothesis has recently received substantial support, since approximately 1 % and 8 % of the mouse and human sperm chromatin, respectively, was found not to be organized by protamines, but associated with sperm nucleosomes that are enriched in genes important for the initial embryo developmental stages (Gatewood et al., 1987; Gardiner-Garden et al., 1998; Zalenskaya et al., 2000; Wykes

& Krawetz 2003; Arpanahi et al., 2009; Hammoud et al., 2009). The rest of the different chromatin-packaging genes is organized in nucleoprotamines (approximately 99 % in mouse sperm and 92 % in human sperm) (Arpanahi et al., 2009; Hammoud et al., 2009). Furthermore, alterations were detected in the gene distribution in nucleoprotamine and nucleohistone domains of infertile patients (Hammoud et al., 2011). Also histone retention is altered in mouse impaired spermatogenesis (Idhara et al., 2014). Therefore, the reported associations between decreased P1/P2 ratios, increased DNA fragmentation and infertility cannot be simply explained by the lack of protection of the chromatin, but may be the consequence of a complex and general sperm chromatin disordering resulting in altered epigenetic imprinting, decreased P1/P2 ratios, altered protamine/DNA content, and increased DNA damage (Carrell et al., 2008; Oliva & Ballesca 2012; Castillo et al., 2014; Jodar & Oliva 2014). Our present results would be consistent with a general sperm chromatin disordering rather than with the classical DNA protection hypothesis. It would be interesting in future studies to measure the effect of TBBPA on the gene distribution in the nucleoprotamine and nucleohistone-packaged regions in sperm and associated DNA methylation patterns.

Although a high variability of P1/P2 has been detected among mammals, a particular proportion of the two protamines seems to be important within the same species (Corzett et al., 2002). Some mammalian species do not have detectable levels of protamine 2 (such as boar and rat) (Oliva & Dixon 1991, Corzett et al., 2002). There are even many vertebrate species with no protamines at all in their sperm cells (such as zebra fish and goldfish), and yet they efficiently manage to transmit intact paternal genomes to the offspring. For example, the zebrafish is a fish species that, instead of protamines, uses increased amounts of linker histone H1 and depletion of chromatin-decondensing modifications (such as H4K16ac) to accomplish sperm chromatin condensation, and where genes important for embryo development are packaged in blocks of multivalent chromatin (Wu et al., 2011). It is interesting to note that different endocrine disruptors have been reported to result in sperm epimutations and increased TUNEL positive cells in the testis of the corresponding animals (Guerrero-Bosagna et al., 2012; Manikkam et al., 2013). The present detection of increased sperm DNA fragmentation in the TBBPA-treated group is also consistent with the recently reported effect of TBBPA in the sperm cells of the starlet fish measured using COMET assay (Linhartova et al., 2014). Increased sperm DNA damage has also been reported for men exposed to other endocrine disruptors such as bisphenol A (Rochester 2013). Of course, the imprinting

hypothesis and the DNA protection hypothesis for protamines are not mutually exclusive and could perfectly both coexist and be valid.

We have not detected any correlation between the P1/P2 alterations present in the sperm and the protamine transcript levels in the testis (Table 2). Therefore, the protamine alterations detected at the protein level in our study are not due to a transcriptional alteration in the corresponding genes, but must have originated through posttranslational processing and/or deposition onto the chromatin. In humans, alterations in protamine transcripts have been described in the testis (Steger et al., 2000; 2001; 2003) and mature sperm of infertile patients (Jodar et al., 2012). Therefore, the mechanisms involved appear to be different in our model from those operating in infertile patients. Separate analysis of the P1/DNA and P2/DNA ratios in the two groups indicates that the abnormally increased P1+P2/DNA ratio in the TBBPA-treated animals is mainly due to a significant increase in the P2/DNA ratio (Fig. 1E) The main difference between the two protamines is that P1 is directly translated into the mature form, while P2 is first synthesized as a precursor and then proteolytically processed to give rise to the mature form of P2 (Torregrosa et al., 2006; de Mateo et al., 2009; 2011). But the increased ratio detected excludes a defect of processing. A potential explanation could be that the transcripts for P1 and P2 are produced normally, but there is some effect of the TBBPA exposure on Prm2 transcript stabilisation or translation that could result in overproduction of P2. The possibility is now open in future studies to further clarify the mechanism involved.

In conclusion, the present work demonstrates that TBBPA exposure results in altered protamine ratios and increased sperm DNA fragmentation. The possibility is now open to further investigation of the molecular mechanisms within the sperm cell through which TBBPA exposure results in the detected effects. Different studies have demonstrated the presence of changes in the sperm proteome in infertile patients linked to protamine imbalances and DNA fragmentation (de Mateo et al., 2007; Martinez-Heredia et al., 2008; Oliva et al., 2008; Amaral et al., 2014). Therefore, important future extensions of our present work will be to explore in detail the overall sperm proteome and epigenome in TBBPA-exposed animals and their offspring, with the potential to provide additional important clues onto the mechanisms involved in the detected alterations. It will also be interesting in the future to conduct studies that limit the exposure of TBBPA to differing stages of development in order to gain further insight into the mechanisms of the detected associations. Another aspect that could be further investigated is to what extent the individual cell-to-cell differences in the protamine content, RNA, and DNA fragmentation could relate to the

detected associations in mixed sperm populations. Finally yet importantly, our results could also emphasize that more attention should be devoted to studying the potential health issues of TBBPA exposure in the human population and particularly those related to reproductive fitness.

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DISCLOSURES

The authors have nothing to disclose.

AUTHORS' CONTRIBUTION

EZ, JP and RO designed the study. EZ, JC, FE, AK and LD performed the bench work. EZ, JC, JP and RO interpreted the data. EZ, and RO wrote the manuscript. All authors critically reviewed and approved the submitted version of the manuscript.

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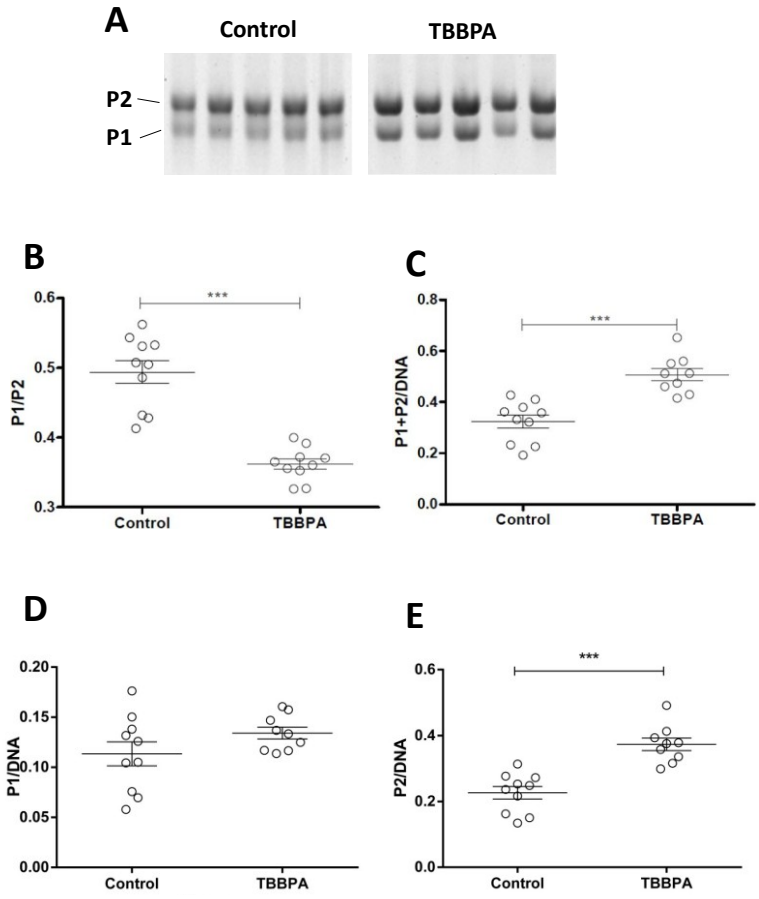

Figure 1

Figure 1: Analysis of protamine 1 (P1), protamine 2 (P2) and protamine ratios in control and TBBPA-treated groups. (A) Representative examples of the visualisation of protamines in mouse sperm samples from control and TBBPA-treated animals. Proteins were extracted from epididymal spermatozoa, separated on acid-urea polyacrylamide gel and stained with EzBlue. Some representative examples are shown. (B) Protamine 1 to protamine 2 ratio (P1/P2) in epididymal spermatozoa. The Mann-Whitney U test shows that the P1/P2 ratio is significantly lower in animals exposed to TBBPA. (C) Protamine to DNA ratio in epididymal spermatozoa. (D) P1/DNA ratio. (E) P2/DNA ratio. The Mann-Whitney U test shows that the P1+P2/DNA ratio is significantly higher in animals exposed to TBBPA. ***P< 0.001. Mean±SD; n=10/group.

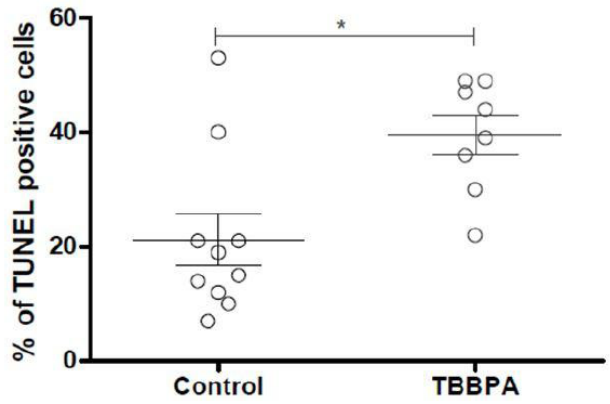

Figure 2

Figure 2: Number of cells positive for terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL expressed as %). The Mann-Whitney U test shows that sperm of animals treated with TBBPA display higher DNA damage compared to the control group (*P< 0.05. Mean±SD; n=10/group).

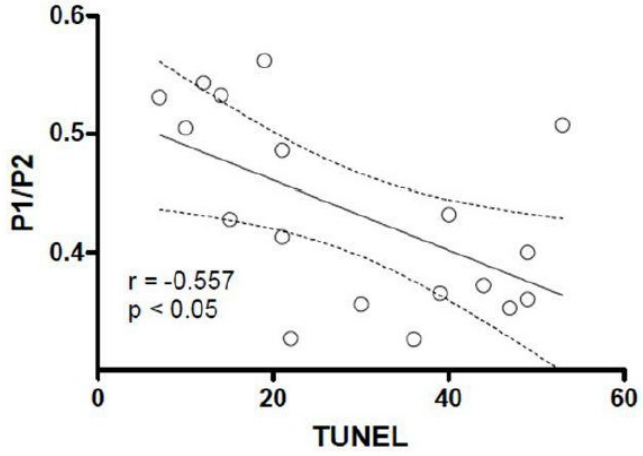

Figure 3

Figure 3: Correlation between DNA damage (number of TUNEL positive cells in %) and P1/P2 ratio. The statistical analysis shows that there is a significant negative correlation between these two parameters (*P < 0.05. Mean±SD; n=10/group).

Gene	Accession no.	Nucleotide sequence 5'-3'	Size of PCR product (bp)
Actb	NM_007393.3	CGGTTCCGATGCCCTGAGGCTCTT	100
		CGTCACACTTCATGATGGAATTGA	
Prm1	NM_013637.4	ACAGGTTGGCTGGCTCGACC	90
		CGGCAGCATCGGTATCTGGCC	
Prm2	NM_008933.1	CCAGGGGCCTGGACAAGACC	112
		TCTGTGGTGGTGGTGGCCCC	
Tnp1	NM_009407.2	CCGAGCTCCTCACAAGGGCGT	140
		CAGGGCAGAGCTCATTGCCGC	
Tnp2	NM_013694.4	CCTGCAAGACCCAGCCACCG	94
		GTTTCCGCCTCCTGACGGCC	

Table1: Primer sequences used in the RT-PCR experiments.

Group	Prm1 (%)	Prm2 (%)	Tnp1 (%)	Tnp2 (%)	Prm1/Prm2
Control	100.0±38.9	100.0±47.2	100.0±26.1	100.0±16.9	0.56±0.10
TBBPA	91.2±28.0	100.1±29.3	100.8±20.5	100.1±16.4	0.60±0.18
P value	0.76	0.59	0.95	0.99	0.66

Table 2: Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis of testicular protamine 1 (*Prm1*); protamine 2 (*Prm2*); transition protein 1 (*Tnp1*) and transition protein 2 (*Tnp*2) genes. Relative expression of the selected genes and the Prm1/Prm2 ratio are given. The control group represents 100 % and the percentage in the experimental group represents the ratio of gene expression between the experimental (TBBPA) and the control group. No significant differences were detected.

Supplementary table: General reproductive parameters and body weight in the control and TBBPA groups

	Control	TBBPA	p-value
AGD [cm]	1,24±0,07	1,27±0,08	**0,35**
Body weight [g]	23,7±2,61	22,4±1,96	**0,26**
Testes [mg]	0,157±0,016	0,148±0,01	**0,19**
Epididymis [mg]	0,073±0,030	0,058±0,008	**0,19**
Prostate [mg]	0,076±0,028	0,076±0,008	**0,99**
Sperm morphology [% of damage]	7,6±2,6	6,1±2,6	**0,21**
sperm concentration [* 10^6 / ml]	17,6±6,4	15,7±5,8	**0,51**

SUPPLEMENT 7

Brieno-Enriquez, M. A,J Garcia-Lopez, D.B. Cardenas, S Guibert, E Cleroux, L Ded, JdD Hourcade, J Peknicova, M Weber and J del Mazo 2014 Prenatal exposure to the fungicide vinclozolin induces transgenerational deregulation of the Lin28/let-7/Blimp1 pathway in mouse germ cells. *Under revision in Cell*.

My contribution to this work: See “Authors' contributions“ in the supplement.

Classification: Biological sciences; Genetics

Prenatal exposure to the fungicide vinclozolin induces transgenerational deregulation of the *Lin28*/*let-7*/*Blimp1* pathway in mouse germ cells.

Running title: Vinclozolin deregulates microRNAs in germ cells

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Keywords: Vinclozolin, microRNAs, Lin28, Blimp1, let-7, PGCs, Transgenerational inheritance, DNA methylation, infertility.

ABSTRACT

In mammals, germ cell differentiation is initiated in the Primordial Germ Cells (PGCs) during fetal development. Prenatal exposure to endocrine disruptors with antiandrogenic activity such as the fungicide vinclozolin may alter the development of the male germline and induce transgenerational epigenetic disorders in the reproductive system. We performed a prenatal exposure to different dosages of vinclozolin in mice and analyzed phenotypic and molecular changes in three successive generations. Herein, we show that vinclozolin induces a long-lasting reduction of the fertility rate and increased apoptosis in adult testis of male mice along three generations. In embryonic testes, we detected a reduction in the number of PGCs associated to an increased rate of apoptotic cells. The *Lin28*/*let-7*/*Blimp1* pathway is involved in the regulation of PGC commitment and differentiation. We observed an upregulation of *let-7* miRNA and concomitant downregulation of *Lin28* expression in PGCs from exposed mice in three generations. Moreover, *miR-23b* and *miR-21*, both implied in the balance of *Lin28-let-7-Blimp1*, are also deregulated. In contrast we found that vinclozolin does not induce prominent changes in DNA methylation in PGCs or mature sperm. Together, our data provide evidence that exposure to vinclozolin, even at low dosage, alters male germ cell development over several generations by, at least in part, deregulating the *Lin28-let-7-Blimp1* pathway.

Significant statement:

Primordial germ cells (PGCs) are the embryonic precursors of oocytes and spermatocytes. Vinclozolin is an endocrine disruptor with antiandrogen activity associated to fertility disturbances in exposed males that can be transgenerational inherited. Herein, we demonstrate that fetal exposure to vinclozolin in mice disrupts a master regulatory pathway of PGC specification (the *Lin28*/*let-7*/*Blimp1* pathway), which is associated to increased rates of apoptotic germ cells in the embryo and adults

as well as decreased number of PGCs and mouse fertility. These phenotypes are transgenerationally transmitted to successive generations of mice through the male germline. This transmission is not associated to major epigenetic alterations of the sperm DNA methylation patterns.

Introduction:

Primordial germ cells (PGCs) are the embryonic precursors of the germ cell lineage, which are restricted to form spermatozoa and oocytes following their specification from pluripotent cells (1). PGC specification depends on the key factors BLIMP1 (also known as PRDM1) and PRDM14 that induce repression of the somatic program, epigenetic reprogramming and re-expression of pluripotency genes. Blimp1 knockout embryos display a low number of PGCs, do not show a migratory phenotype and are unable to properly express PGC-specific genes (2, 3). The formation of PGCs also requires the RNA-binding factor LIN28 that binds to specific microRNA (miRNA) precursor: the let-7 pri-miRNA preventing the processing into mature forms of let-7 miRNAs. In absence of LIN28, let-7 miRNAs are overexpressed in PGCs and bind to the 3□ ŚUTR of the Blimp1 mRNA, which blocks its translation and inhibitsPGC development (4).

In mouse, PGC precursors are specified in the epiblast around 6.25 days post coitum (dpc) (5). Thereafter, PGCs proliferate and migrate through the hindgut endoderm to enter the genital ridges at day 10.5 and colonize the fetal gonads where they continue to proliferate until day 13.5 (6). During this period, PGCs undergo global epigenetic reprograming characterized by the erasure of DNA methylation and histone modifications (7). After the onset of gonadal sex determination, the PGC genome initiates re-methylation of DNA accompanied by remodeling of histone modifications in a sex specific manner (7, 8). Genetic and epigenetic changes during reprogramming of embryonic germ cell precursors make the prenatal period a sensitive window for potential adverse effects caused by environmental factors. The environmentally induced

changes produced at this period are capable of inducing adult onset diseases than can also be perpetuated across multiple generations by transmission through the germ line (transgenerational epigenetic inheritance) (9). Epigenetic mechanisms, including DNA methylation, histone modifications and specific miRNAs expression have been proposed to mediate such transgenerational transmission (10, 11).

Endocrine disruptors (EDs) are synthetic or natural substances that alter the homeostasis of the endocrine system and produce adverse developmental, reproductive, neurological, behavioral and immune effects in humans and wildlife animals. The effects of EDs vary in relation to the nature of the compound, the dose, and the period of exposition during development. A famous example of ED influencing germline functions in mammals is Vinclozolin (VCZ) (3-(3, 5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-

2, 4-dione), an antiandrogenic fungicide used on vineyards, fruits, vegetables and ornamental plants. VCZ metabolites are competitive antagonists of androgen receptor (AR) ligand binding (12). Several studies performed in rodents (mainly rats) showed that exposure to VCZ during the gestational period of gonadal sex determination induces masculinized females, feminized males (13), decreased anogenital distance (14), decreased sperm number and increased apoptosis in the seminiferous tubule cells, (15), and abnormal fertility rates (16). Some of the effects of VCZ have been observed to be passed on transgenerationally to subsequent unexposed generations, which are hypothesized to be caused by the gametic transmission of deregulated epigenetic marks such as altered DNA methylation (15, 17-19).

Environmental exposure to chemicals can induce aberrant miRNA expression (20). miRNAs are small non-coding RNAs (~21-23 nt) participating in posttranscriptional gene silencing of target mRNAs (21). Some studies have established that miRNAs can also influence epigenetic regulation by interacting with DNA methylation or the deposition of repressive histone modifications (22).

In the present study, we analyzed by an integrative approach the effects of prenatal exposure to VCZ in mice. We evaluated the effects of VCZ in the first generation of exposed animals as well as the transgenerational transmission through the male germline in subsequent generations (F1 to F3). We monitored several phenotypic and functional parameters in embryonic gonads and adult testis, and related these traits to the profiles of miRNA expression and DNA methylation in germ cells. We describe that prenatal exposure to VCZ induces a perturbation of apoptosis and fertility that persist over three generations in male mice. We provide evidence that this phenotype is associated with the deregulation of several miRNAs in PGCs, in particular the Lin28/let-7/Blimp1 pathway that plays important roles in PGC specification, but no major changes in DNA methylation. Our study uncovers a new molecular pathway involved in the lifelong effects caused by endocrine disruptors in mammals.

Results

Transgenerational phenotypic consequences of prenatal exposure to vinclozolin in males

Pregnant females were exposed to VCZ by oral intake (in the drinking water) during the entire duration of pregnancy with two different doses: a low dose (1mg/kg bw/d) (VD1) and a high dose (100mg/kg bw/d) (VD2). The offspring obtained from these exposed females were denominated F1 animals. F2 animals were obtained from the mating of F1 males with unexposed females, and F3 animals from the mating of F2 males with unexposed females (*Material and Methods* and Fig. S1).

We first performed a global phenotypic analysis of adult male mice in all three generations (F1 to F3). We found no differences in body weight, testis weight, and other morphometric markers between VCZ exposed and control animals (data not shown). However, we observed that the male fertility rate is reduced by 8% in VD1 and 12% in VD2 F1 males compared to the control group (Fig. 1A). The fertility rates of animals of the VD1 group recovered to values comparable to non-exposed control animals along

the next generations (F2 and F3). In contrast, the males from the high dosage (VD2) group showed a stable reduction of 12% in their fertility index in all the generations analyzed (F1-F3) (Fig. 1A).

To evaluate whether the reduction in the fertility index was associated to phenotypic changes in the testis, we monitored histopathological anomalies and apoptosis in the seminiferous epithelium from adult testis from F1 to F3. Histological analysis showed a tendency towards increased number of impairment of seminiferous epithelium tubule and hypertrophic cells with fragmented karyoplasm in the lumen of tubules in the three generations of exposed males (Figs. 1B-E), however the differences observed were not statistically significant. Nevertheless, when we evaluated the number of apoptotic cells by the TUNEL method, we detected a statistically significant increase of apoptosis into seminiferous tubules of adult testis in all the generations and with both doses (Figs. 2A and 2B-E) ($p \leq 0.0001$). Surprisingly, apoptosis is increased to similar levels (1,38 to 1,50 fold compared to control animals) in males exposed to low dose (VD1) and high dose (VD2).

To determine if gonadal defects in exposed animals are a consequence of dysfunctions that arise in embryonic germ cells, we analyzed PGCs isolated at 13.5 dpc using the surface marker SSEA-1 (Material and Methods). The results revealed that VCZ exposure induces a decrease in the number of PGCs recovered per testis. Compared to controls, F1 animals from the VD1 and VD2 groups showed a statistically significant reduction of 57% and 35% respectively (p.0.001) in the number of PGCs (Fig. 2F). The reduction in the PGC number was similar in F2 males with a reduction of 61% in VD1 and 38% and VD2 (p.0.001) (Fig. 2F). However, the number of PGCs recovered to normal levels in both the VD1 and VD2 group in the F3 generation (Fig. 2F). To corroborate these findings, we performed immunofluorescence with the marker SSEA-1 in sections of 13.5 dpc testis. The number of positive SSEA-1 signals per unit area is significantly lower in F1 and F2 13.5dpc testis from exposed animals compared

to the unexposed controls, and restored to normal values in F3 (Fig. 2G). In summary, both experimental approaches indicated a significant reduction in the number of PGCs at 13.5dpc in F1 and F2 males exposed to VCZ.

To uncover the potential differences in the PGCs apoptotic rate among control and experimental samples, colocalization analysis of the TUNEL and SSEA-1 positive cells (Figs. 2I-K) were performed using NIS elements picture analyzer. The signals from corresponding TUNEL and SSEA-1 fluorescence channels in individual testis embryo sections were measured and the correlation of the fluorescent signal intensity was computed. The results were outputted as Mander's overlap (MO) coefficient and Pearson's correlation coefficient (r) (23). The results showed that there was a significantly higher overlap (MO) of the TUNEL and SSEA-1 fluorescent signals in three experimental groups with the higher exposition to VCZ compared to the control group. The results showed a decrease of positive signals in F1-VD1 and VD2 as well as in F2VD2 (Fig. 2H). The mean value of Rp- Pearson□ fs correlation (Rp) in control was 0.72; representing that 51% of the apoptotic signals (TUNEL) were coincident with SSEA-1 signal. Testis from VCZ exposed mice showed a significantly increment, reaching the maximum in F1VD2 (Rp=0. 90) representing that 80% of signal colocalized. These results indicate that PGCs from experimental groups exposed to VCZ have significantly higher probability to undergo apoptotic process compared to the PGCs from non-exposed animals.

Prenatal exposure to vinclozolin induces a transgenerational perturbation of miRNAs involved in PGC differentiation

Using miRNA TaqMan-Megaplex arrays (Applied Biosystems) interrogating 518 mouse miRNAs, we profiled the differential expression of miRNAs in PGCs from VCZ-exposed and control F1 mice and identified several deregulated miRNAs (data not shown). After, we selected candidate miRNAs owing to their reported or predicted role in germ cell development and differentiation. This includes members of the let-7 family

and miR-23b, which can regulate Blimp1, a key in the PGC differentiation (2, 24, 25). let-7 and mir-23b also regulate Lin28, which encode a stem cell-expressed RNA binding protein required for PGC development. In turn, LIN28 establishes a feedback loop by binding to let-7 pre-miRNAs and pri-miRNAs and inhibiting their processing into mature miRNAs (26-28). In mammals, two Lin28 paralogs are expressed (Lin28a and Lin28b), which are functionally equivalent but with different expression patterns and cellular localization, and they repress let-7 by distinct mechanisms (29). We first quantified the expression levels of let-7 miRNA and target mRNAs in samples from all three generations. Our results showed that exposure to VCZ increased significantly the expression of the precursor and some mature forms of let-7 in F1 to F3 (p.0.001) (Figs. 3A-B). The increased levels of let-7 in PGCs correlate with a downregulation of the Lin28 paralogs. We found that Lin28a is downregulated in PGCs from exposed F1 and F2 animals, whereas its expression was slightly increased in F3 in an unexpected rebound-like effect. Similarly, Lin28b expression levels are decreased in PGCs from F1 and F2 animals (p.0.001), whereas the levels tend to normalize in F3 (Figs. 3C-D). To corroborate these findings at the protein level, we performed Western blot analysis of LIN28 in PGCs and observed a decrease of protein levels in F1 and F2 exposed animals and a recovery to normal levels in F3 (Fig. 3E), which agrees with the profiles detected for the Lin28a and Lin28b transcripts. In addition to let-7, Lin28 is also a predicted target of miR-23b. We found that miR-23b is also upregulated in a dosage-dependent manner in PGCs from the three generations of embryos exposed to VCZ (Fig. 4A).
In addition to Lin28, let-7 and miR-23b are also potential regulators of Blimp1. Therefore we next asked whether the changes in miRNA expression in PGCs are associated with changes in the expression of Blimp1. We quantified Blimp1 mRNA by RT-qPCR and found strong downregulation of Blimp1 transcripts (p.0.001) in PGCs from the F1 animals exposed to VCZ (Fig. 4B). The reduction of *Blimp1* expression is also transmitted to PGCs of the F2 and F3 generations, even though it tends to revert to

normal levels (Fig. 4B). In accordance with the mRNA levels, we confirmed by Western blot analysis that the protein levels of BLIMP1 were decreased in PGCs of the F1 to F3 generations from F1 exposed animals (Fig. 4C). Altogether, our data show that the transgenerational upregulation of *let-7* and *miR-23b* in PGCs of VCZ exposed animals is associated with the downregulation of the key PGC factors *Lin28* and *Blimp1* along three generations.

To explore whether other miRNAs and genes involved in germ cell development are altered in PGCs as consequence of VCZ exposure, we examined the expression of *miR-21*, *miR-135**, *miR-381* and *miR-486* miRNAs, along with bone morphogenetic protein 4 (*Bmp4*) and signal transducers *Smad*s (*Smad1*, *4*, and *5*) mRNAs from F1 to F3. The miRNA *miR-21* has been implicated in the regulation of spermatogonia stem cells in mice (30) and its transcription is regulated by BLIMP1.(31). We found that VCZ led to elevated *miR-21* levels in PGCs from F1 and F2 but not F3 animals (Fig. 4D). For the other miRNAs and genes analyzed, the data obtained did not allow us bring out a perturbation of these pathways in germ cells of exposed animals (data not shown).

Finally, terminal uridylyl transferases (TUTases) interact with LIN28 to induce poly-uridylation of *let-7* precursors at their 3' end, which interferes with *let-7* maturation and facilitates the miRNA precursor degradation by the recruitment of exonucleases (32). *TUTase4* (*Zcchc11*) and *TUTase7* (*Zcchc6*) also promote the monouridylation of *pre-let-7* in absence of LIN28, which facilitates DICER processing and increases the levels of mature *let-7* (33). To investigate whether the deregulation of the *Lin28*/*let-7* pathway detected in PGCs is associated to the deregulation of TUTases, we performed RT-qPCR analysis of *TUTase4* and *TUTase7*. Surprisingly, both genes were found significantly overexpressed in PGCs of VCZ-exposed mice in the three generations ($p \leq 0.001$) (Fig. S2). Together, these results suggest that a coordinated deregulation of *Lin28* and TUTases participate in the upregulation of *let-7*.

Altogether, these results indicate that in the mouse, the exposure to VCZ, even at low dosage, affects male fertility, apoptosis in adult testis and PGC development over three generations. We demonstrate that VCZ exposure disturbs the expression of several miRNAs and the balance of the *Lin28/let-7/Blimp1* pathway during the specification of PGCs, which provides a possible molecular explanation for the perturbation caused in germ cell precursors (Fig. 5).

Impact of vinclozolin exposure on DNA methylation profiles in PGCs and spermatozoa from F1 males

Previous studies performed in rodent models proposed that the transgenerational effects caused by environmental factors such as VCZ exposure are mediated by the transmission of altered gametic DNA methylation (15, 18, 19). To determine whether vinclozolin influences patterns of gametic DNA methylation in our experimental model, we generated global maps of CG methylation using the reduced representation bisulfite sequencing (RRBS) technique. Using this technique, we obtained quantitative estimates of methylation at more than 8x sequencing depth for more than 1,300,000 CpGs, located mostly in regulatory CpG-rich sequences. We first asked whether VCZ interferes with epigenetic reprogramming in PGCs by generating RRBS maps in 13.5 dpc PGCs from control and exposed F1 males. As expected, control PGCs showed a massive erasure of DNA methylation characteristic of these cells (7). However, this global demethylation pattern is not altered in PGCs from F1 males exposed to the low dose or the high dose of VCZ (Fig. 6A). The rare sequences that retain methylation in control PGCs, which are mostly associated with transposable elements, have similar methylation in PGCs from exposed animals (Fig. 6B). We investigated methylation at genes involved in germ cell function and development and found no evidence for abnormal methylation in PGCs from exposed animals, including in the promoters of genes deregulated in PGCs (*Lin28a*, *Lin28b*, *Blimp1*) (Fig. 6C). Taken together these results indicate that VCZ does not interfere with the process of DNA methylation

reprogramming in early PGCs in our experimental model and deregulates PGC developmental pathways independently of DNA methylation.

We next asked whether abnormal DNA methylation accumulates during later stages of spermatogenesis in male mice exposed to VCZ. We focused on F1 males exposed to the high dose of VCZ because this dose induces the strongest transgenerational phenotype (see above). We generated RRBS methylomes in spermatozoa isolated from exposed F1 males and several control pools (see methods). We observed identical global profiles of CG methylation in control and exposed animals, indicating that VCZ has a minor impact on sperm methylation (Fig. 6D). We identified only very few sequences with variable methylation between samples, most of them being already variable in control samples and therefore representing natural epigenetic variations (data not shown). We only identified one sequence with reduced methylation in exposed compared to control samples, located in the exon 18 of the *Paok3* gene (Fig. S3A, Table S1). Interestingly, it was reported that this gene can be regulated by androgen in human cells (34). In contrast to results obtained with another model of mice exposed to VCZ (17), we found no evidence that VCZ modifies DNA methylation at sequences carrying long term epigenetic memory such as imprinting control regions (Fig S3B). Therefore our data indicate that the exposure to VCZ does not induce major changes in gametic DNA methylation, suggesting that mechanisms other than DNA methylation participate in the transgenerational transmission of VCZinduced phenotypes.

Discussion

This work demonstrates that the fetal exposure of mice to VCZ induces transgenerational changes in miRNAs and target gene expression profiles of the *Lin28/let-7/Blimp1* pathway in PGCs, even at a dosage under those considered as "No Observed Adverse Effect Level." (NOAEL) established as 6 mg/kg/day for "accute dietary

NOAEL" and 1.2 mg/kg/day for the "Chronic (Non-Cancer) Dietary NOAEL" (http://www.epa.gov /oppsrrd1/REDs/2740red.pdf).

Embryonic and early postnatal exposure to endocrine disruptors has been shown to promote adult onset disease in numerous species. Anway et al. first described that intraperitoneal exposure of female rats to VCZ during gestation (day 8-15) induced increased spermatogenic cell apoptosis, decreased sperm count and motility, histological abnormalities and altered DNA methylation patterns along four generations. In the present study, VCZ was administered orally in the drinking water to pregnant female mice until delivery. Using this experimental protocol, we show that mice exposed to VCZ have an increased rate of apoptosis in adult testis in three generations as well as a reduction in the fertility as was described previously in rat (15). This agrees with previous results obtained in mouse models exposed to VCZ (14, 18, 35). In contrast, we did not observe differences in weight, sperm number or other histological patterns in adult offspring mice. These differences with respect to previous studies might be related to the route of administration, the species and the strain used. The increased incidence of apoptosis in adult testis could be the consequence of deregulations detected in early PGCs, potentially caused by imbalanced signals between the inhibitory effects of androgens in fetal germ cells (36) and the antiandrogenic action of VCZ. Interestingly, the phenotypic consequences of exposure are observed with both doses of VCZ and in most cases do not show a dose-response effect. The exception is the fertility rate that is more impaired with the high dose compared to the low dose (Fig 1A). These results provide further support to the theory that the exposure to environmental toxicants during fetal development can induce dysfunctions and diseases in adulthood and even in the next generations, the so-called fetal origin of adult diseases (37).

MicroRNAs modulate gene expression and play key roles in developmental processes, regulation of cell self-renewal, cellular differentiation, proliferation, and

apoptosis. However, the functional importance of miRNAs in the toxicity caused by VCZ has not been investigated. Our results show that VCZ disrupts the normal pattern of expression of specific miRNAs and some of their putative target genes in germ cell precursors. Deregulated miRNAs (*let-7*, *miR-21*, *miR-23b)* and their targets genes (*Lin28a*/*Lin28b* and *Blimp1*) are involved in PGC specification and development, therefore we speculate that this contributes to abnormal germ cell development in exposed embryos. BLIMP1 is responsible for the specification of PGCs in the posterior epiblast mediated by the expression of *let-7,* in turn regulated by LIN28 (24, 38). Therefore, balance among *Blimp1*, *Lin28* and *let-7* needs to be tightly controlled for the proper specification of PGCs in the mouse embryo. Our results demonstrate for the first time that VCZ induces a deregulation of the *Lin28/let-7/Blimp1* pathway in embryos. We observed a downregulation of the abundance of *Lin28* transcripts and consequently also the LIN28 protein in PGCs. As was described in *Lin28* knockdown mice (39), the reduction of LIN28 by VCZ is associated with an accumulation of precursor and mature forms of *let-7* in germ cells. At the same time, VCZ also induces the overexpression of *TUTase4* (*Zcchc11*) and *TUTase7* (*Zcchc6*) that, in absence of LIN28, facilitate the processing of *pre-let-7* into *let-7* (33), which can also contribute to the accumulation of mature *let-7* in exposed animals. In accordance with the model of *Blimp1* regulation, we show that the downregulation of *Lin28* and upregulation of *let-7* are associated with a downregulation of *Blimp1* in PGCs from exposed animals. The changes observed in PGCs of mice exposed to VCZ mimic the changes observed in *Blimp1* heterozygous mutants where the partial reduction of *Blimp1* expression leads to a smaller number of PGCs in embryos which fail to show the characteristic migration and proliferation of PGCs (2, 3). This leads us to propose that the deregulation of the *Lin28/let-7* pathway and partial reduction of *Blimp1* is one molecular pathway that directly contributes to inhibit PGC development in embryos exposed to VCZ (Fig. 5).

Interestingly, previous studies showed that *let-7* is overexpressed after exposure

to other EDs. For example, Bisphenol A (BPA) induces the overexpression of three members of *let-7* miRNA family in human placental cells (40). As mentioned, *let-7* family members and *Lin28* are critical regulators of cell proliferation and are considered as tumor suppressors (26, 41, 42). In this sense, one provocative idea could be that the upregulation of *let-7* miRNAs is a cellular response to protect from the damage induced by environmental factors.

Besides the *Lin28/let-7/Blimp1* pathway, other pathways certainly also contribute to the defects in PGC development and apoptosis caused by the exposure to VCZ. Our results show that VCZ induces an upregulation of *miR-21* and *miR-23b* in PGCs. *miR-23b* is a potential regulator of *Lin28* and *Blimp1*, but at the same time previous reports indicate that *miR-23b* is also involved in the control of cell migration (43), cell adhesion (43), growth arrest (44) and apoptosis (45). On the other hand, *miR-21b* is important for the self-renewal of mouse spermatogonial stem cells. (30). Studies performed in human breast cancer cells showed that cells exposed to other fungicide with antiandrogens effects also overexpress *miR-21*, which reduces cell proliferation and motility (46). This suggests that other miRNAs such as *miR-23b* and *miR-21* could participate in the response to toxic effects of VCZ by modulating cell proliferation and migration in germ cells.

The consequences of VCZ exposure on germ cell and gonadal functions are not only observed in F1 animals exposed *in utero*, but are transmitted through the male germline to subsequent unexposed generations in F2 and F3. We note however that several of the phenotypic traits and gene markers tend to revert to a normal state in F3, which is indicative of an incomplete inheritance. This can be viewed as an example of intergenerational and transgenerational epigenetic inheritance in the mouse (47). Alternatively, given that we used a model of outbread CD-1 mice, we cannot formally exclude the possibility of a gametic genetic selection induced by VCZ. Genetic mutations induced by VCZ or by reactivated transposable elements can also not be

excluded.

What could be the mechanisms of epigenetic transmission through generations? Previous studies have proposed that the epigenetic inheritance of phenotypes is mediated by the transmission of abnormal gametic DNA methylation (15, 18, 19). In the mouse, the primordial germ cells undergo a global wave of epigenetic reprogramming during development that restores their pluripotency, which includes the erasure of somatic profiles of DNA methylation and histone posttranslational modifications (7, 48). In the male germline, PGCs then re-establish DNA methylation de novo and acquire the methylation profiles characteristic of mature spermatozoa between 13.5 and 17.5dpc. In the present study, we show that PGCs from F1 embryos exposed to VCZ have normal patterns of DNA methylation when we interrogated 1,300,000 CpGs by RRBS, which indicates that VCZ does not interfere with the global process of DNA methylation reprogramming in PGCs. Recent reports identified a small number of sequences that escape demethylation in PGCs, which are mostly associated with transposable elements (7, 49). These sequences represent prime candidates for epigenetic transgenerational inheritance, however we found that their methylation is not dramatically altered in PGCs from exposed embryos. We also searched for patterns of abnormal DNA methylation in mature spermatozoa and found a very strong conservation of cytosine methylation marks in spermatozoa of VCZ-exposed males. Several explanations can be proposed to explain this result. First, it is possible that changes in DNA methylation occur outside of the genomic regions covered by the RRBS method. RRBS covers the majority (>95%) of CpG islands found in regulatory regions in the genome, but it only provides a superficial coverage of CpG-poor sequences in intragenic and intergenic regions that can also influence gene expression states. Second, we cannot rule out that changes in DNA methylation of small amplitude, possibly occurring in some spermatozoa cells or some individuals only, are sufficient to drive the observed transgenerational phenotypes.

Alternatively, epigenetic marks other than cytosine methylation could mediate the trans-generational effects observed in VCZ-exposed mice, for example changes in the patterns of histones modifications. Recent reports have shown that the mouse spermatozoa retain histones carrying specific marks of histones. This suggests that sperm modified nucleosomes, in particular those methylated on the lysine 27 of histone H3 (H3K27me3), could mediate paternal epigenetic inheritance and influence gene expression in the embryo (50). This model is also supported by a recent bioinformatics modeling approach that predicts that histone modifying enzymes play an important role in the response to endocrine disruptors such as vinclozolin and dibutyl-phthalate (51). Another possibility is that the epigenetic transmission is mediated by sperm miRNAs. It has been described that miRNAs can induce hereditary epigenetic variations in mice and act as the transgenerational signalling molecule. Two examples of this mechanism called "paramutation" include the epigenetic modulation of the *Kit* gene and *Sox9* gene in mice (52, 53). More recently, new studies implicated sperm small RNAs in the transgenerational transmission of paternal stress in mice (54, 55). Given that we find that a number of miRNAs are disregulated in germ cells as a consequence of exposure to VCZ, the possible role of miRNAs in the paternal transgenerational transmission of ED-induced phenotypes is a plausible mechanism of action that needs to be investigated in the future.

Material and Methods

Detailed material and methods can be found in *S1 Materials and Methods*.

Mice

All procedures for handling CD-1 mice were in accordance with the regulations of the European Commission (directive 2010/63/UE) and in accordance with protocols approved by the CSIC Bioethics Committee.. CD-1. Mice were exposed to VCZ by oral

intake in drinking water. Three groups were established: control group (exposed to vehicle: DMSO), vinclozolin low dose group (VD1, 1 mg/kg bw/d) and vinclozolin high dose group (VD2, 100mg/kg bw/d) (Fig. S1). Detailed procedures in ***S1 Materials and Methods***

PGC isolation

PGCs were purified using paramagnetic technology from 13.5 dpc male embryo testes following a published protocol (56) with some modifications detailed in ***S1 Materials and Methods***. In all cases the purity of the PGCs was 93-96%.

Histological analysis and adult testicular tissue morphometry

From each adult mouse, one testis was fixed in 4 % formaldehyde in PBS.

Paraffin-embedded tissue sections (5µm thick) were stained by hematoxylin-eosine. Tissue sections were. For each testis, 100 seminiferous tubules were analysed under a light microscope by computer-assisted morphometry. The thickness of the germinal epithelium and diameter of the seminiferous tubules were measured.

Apoptotic cells and immunocytochemistry analyses

Apoptotic cells in tissue sections were assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), using an in situ detection kit (Promega) according to the manufacturer's instructions. Immnicytochemical analysis of PGCs in embryo testes were performed labelling by anti-SSEA1 antibody (Abcam, ab16285) and TUNEL as described in S1 Materials and Methods.

miRNA and mRNA analyses

Total RNA was isolated from whole testes and PGCs using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and resuspended in RNasefree

water. The concentration was estimated by absorbance (A260/280 ratio) on a NanoDrop ND-1000 spectrophotometer (NanoDrop). RNA integrity was verified using an Agilent Bioanalyzer 2100 (Agilent). miRNA expression analysis were performed by reverse transcription followed by quantitative PCR (RT-qPCR) using specific stem-loop primers and TaqMan probes (Applied Biosystems). mRNA expression were analyzed by stadard procedure using the expression of Sdha and Mapk1 as normalizer genes. Detailed procedures and primers are described in S1 Materials and Methods.

Western Blot Analysis

Protein extracts were prepared from PGCs by one cycle of freeze-thaw lysis in RIPA buffer supplemented with 1x antiprotease cocktail, 250 mM NaF and 100 mM Na3VO4 (Sigma-Aldrich). Proteins were anlysed by standard Western Blot procedures and detailed in S1 Materials and Methods.

Methylation analysis.

RRBS was performed on 100ng of genomic DNA prepared from PGCs or spermatozoa as described (57). Gene-specific analysis of DNA methylation, were performed by bisulphite conversion of genomic DNA. Detailed procedures for DNA methylation are described in S1 Materials and Methods

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J.G-L., D.B.C., S.G., L.D., JdD. H. performed research; M.A.B-E., J.G-L., E.C., J.P., M.W and J.dM. analyzed data; and M.A.B-E., J.G-L, J.P., M.W. and J.dM. wrote the paper.

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Figure legends

Figure 1. **Fertility and histopathological analysis in the testis of mice exposed to VCZ. A)** Fertility rate after fetal exposure to the low dose (VD1) or the high dose (,VD2) of VCZ, expressed as a percentage of fertile males along the three generations (F1, F2 and F3). **B-E)** Histological analysis of testis sections stained by hematoxilyneosin from 10 weeks old mice from the control group (B) or VCZ exposed group (C-E), show examples of impairment of seminiferous epithelium tubule (C), tubule disintegration with cells in the lumen (D), and hypertrophic cells with fragmented

karyoplasm (E, red arrows).

Figure 2. Apoptosis in adult testis and PGCs of mice exposed to VCZ. **A)** Number of apoptotic cells counted per 10 tubules in the adult testis of control, VD1 and VD2 in F1 to F3 generations.. **B-C)** Examples of apoptosis by TUNEL in testis sections of control **(B**), VD1 (**C**), VD2 (**D**) and TUNEL positive signals after DNAse treatment of the sections (positive control) **E). F)** Total number of PGCs isolated per testis by cell sorting with the surface marker SSEA-1 from control, VD1 and VD2 13.5dpc embryos. **G)** Histological evaluation of the number of PGCs in testis of 13.5dpc embryo by immunostaining with the marker SSEA-1. **H)** Evaluation of apoptosis in 13.5 dpc testis by TUNEL assay. **I-K)** Examples of confocal microscopy analysis of apoptosis, and immunofluorescence SSEA-1 positive PGCs cells. **I**) Apoptosis detected in a PGC. J) Apoptosis detected in a somatic cell. **K**) None apoptotic cell detected. In the histograms, (a) significant statistical differences compared to control, the bars include standard deviation (SD).

Figure 3. **Expression of *pri-let-7a*, *let-7a-1-3p* and *Lin28* in PGCs of mice exposed to VCZ** along the three generations. **A-D**) The graphs show the log2 of the fold change of expression in exposed embryos relative to the unexposed control embryos. **E**) LIN28 protein levels measured by Western blot in 13.5 dpc PGCs. The graph bars show the quantification of protein levels normalized to the unexposed control (value=1). The graph bars include standard deviation (SD), (a) indicates statistical differences of VD1 and VD2, compared to control ($p \leq 0.01$), (b) indicates statistical difference of VD1 compared to VD2 ($p \leq 0.01$). The bars include standard deviation (SD).

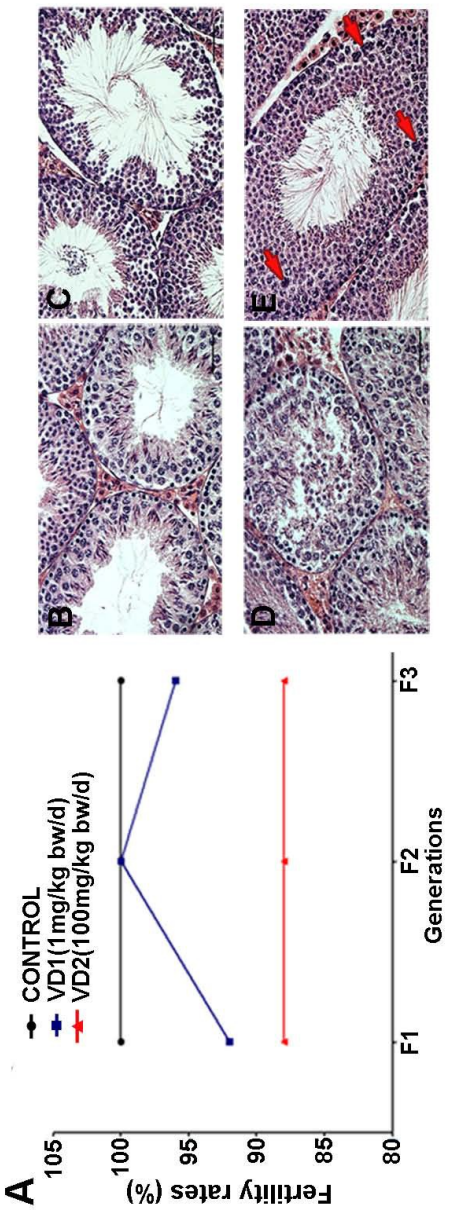
Figure 4. **Levels of expression of *Blimp1* and interacting miRNAs in PGCs A**) Relative expression of *miR-23b*. **B** and **C**) Relative expression respect to controls of *Blimp1* mRNA and protein. **D**) Relative expression of *miR-21*. The graph bars include standard deviation (SD), (a) indicates statistical differences of VD1 and VD2, compared to control ($p \leq 0.01$), (b) indicates statistical difference of VD1 compared to VD2

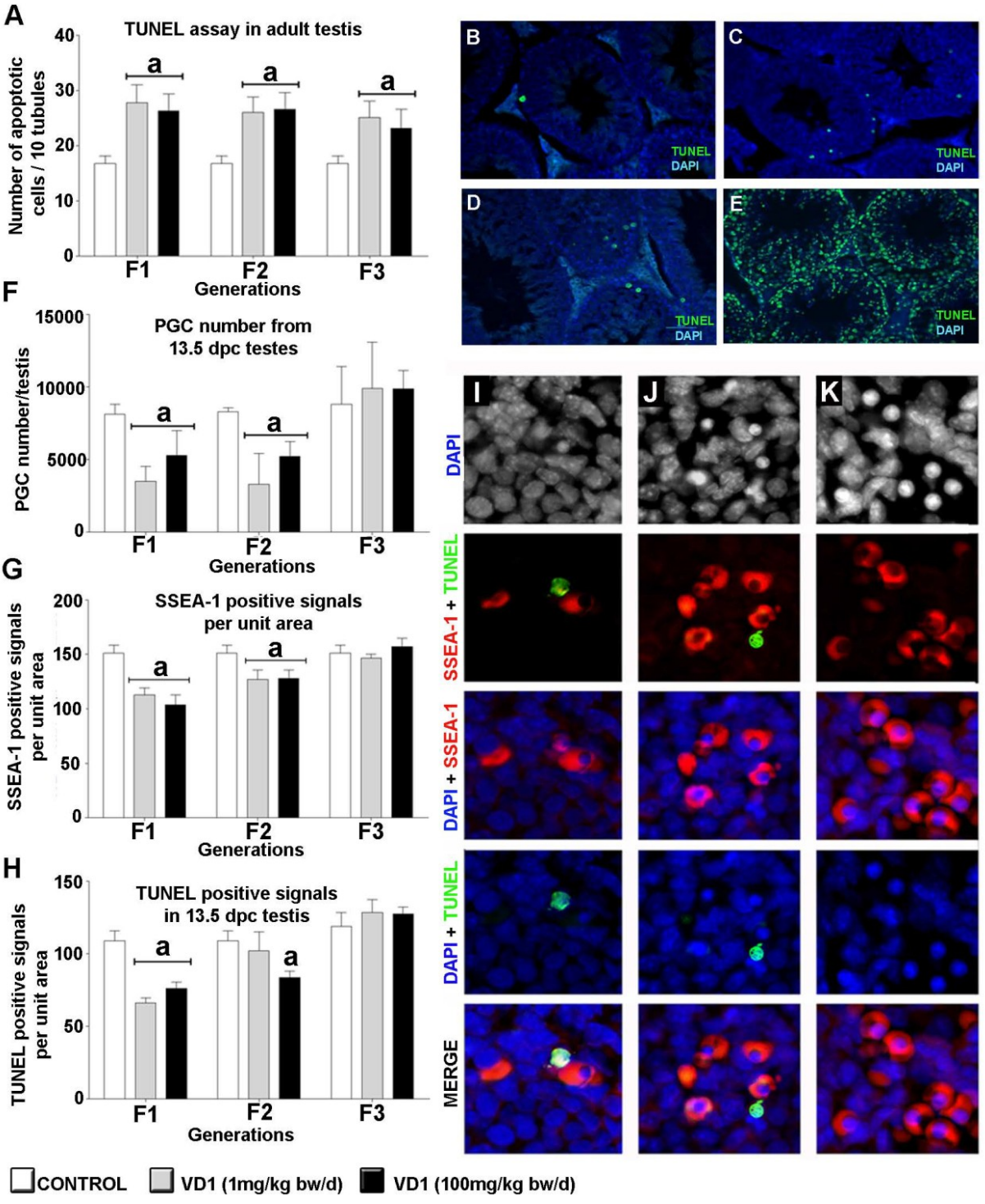
($p \leq 0.01$).

Figure 5. **Schematic model for the deregulation of PGC development by the *Lin28*/*let-7*/*Blimp1* pathway in the PGCs from mice exposed to VCZ**. Red arrows indicate upregulation and green arrows indicate downregulation. miRNAs are illustrated in pink. A consequence of the downregulation of BLIMP1 is the reduction in the number of PGCs in embryonic testis.

Figure 6. **DNA Methylation analysis in PGCs and spermatozoa of F1 mice exposed to VCZ**. **A**) Distribution of the percentages of CpG methylation measured by RRBS in 13.5 dpc PGCs from control, VD1 and VD2 exposed embryos at the F1 generation. **B**) Distribution of the CG methylation measured in sequences hypomethylated in normal PGCs (defined as <20% methylation in control PGCs) or partially resistant to demethylation in PGCs (defined as >20% methylation in control PGCs). **C**) Evaluation of the methylation status of the promoters of *Lin28a*, *Lin28b* and *Blimp1* in 13.5dpc PGCs by single locus bisulfite sequencing (for *Lin28a* and *Lin28b*) and RRBS (for *Blimp1*). **D**) Pairwise comparison of CpG methylation measured in 400bp tiles in adult spermatozoa isolated from F1VD2 males compared to control males, which reveals global conservation of DNA methylation. The density of points increase from blue to dark red.

Figure 1


A
105
100
95
90
85
80
Fertility rates (%)
CONTROL
VD1(1mg/kg bw/d)
VD2(100mg/kg bw/d)
F1
F2
F3
Generations
B
C
D
E


A
TUNEL assay in adult testis
Number of apoptotic cells / 10 tubules
40
30
20
10
0
a
a
a
F1
F2
F3
Generations
B
C
D
E
TUNEL
DAPI
F
PGC number from 13.5 dpc testes
PGC number/testis
15000
10000
5000
0
G
SSEA-1 positive signals per unit area
SSEA-1 positive signals per unit area
200
150
100
50
0
H
TUNEL positive signals in 13.5 dpc testis
TUNEL positive signals per unit area
150
100
50
0
I
J
K
DAPI
SSEA-1 + TUNEL
DAPI + SSEA-1
DAPI + TUNEL
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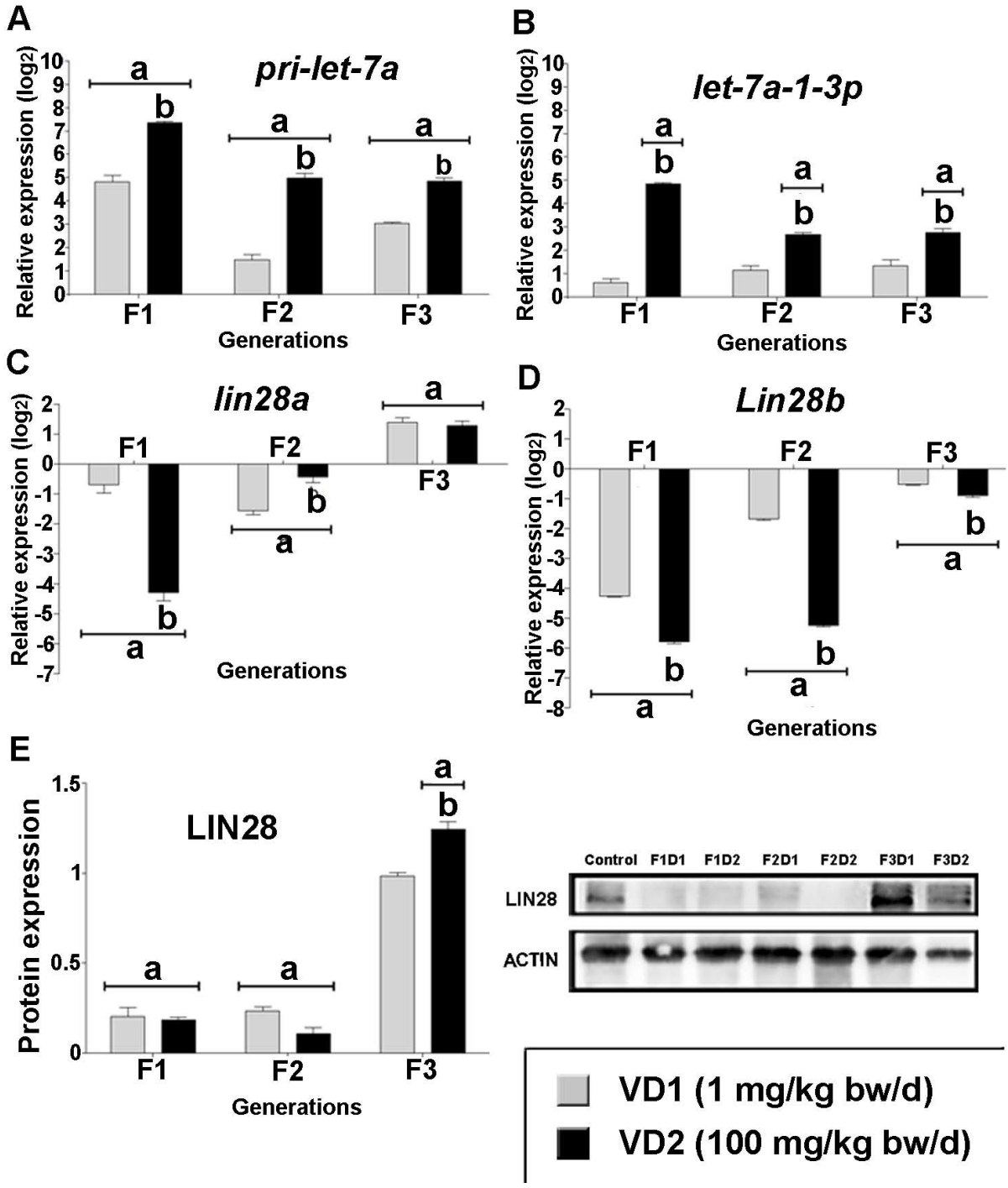
VD1 (100mg/kg bw/d)


A
pri-let-7a
Relative expression (log2)
Generations
F1
F2
F3
B
let-7a-1-3p
C
lin28a
D
Lin28b
E
LIN28
Protein expression
Control
F1D1
F1D2
F2D1
F2D2
F3D1
F3D2
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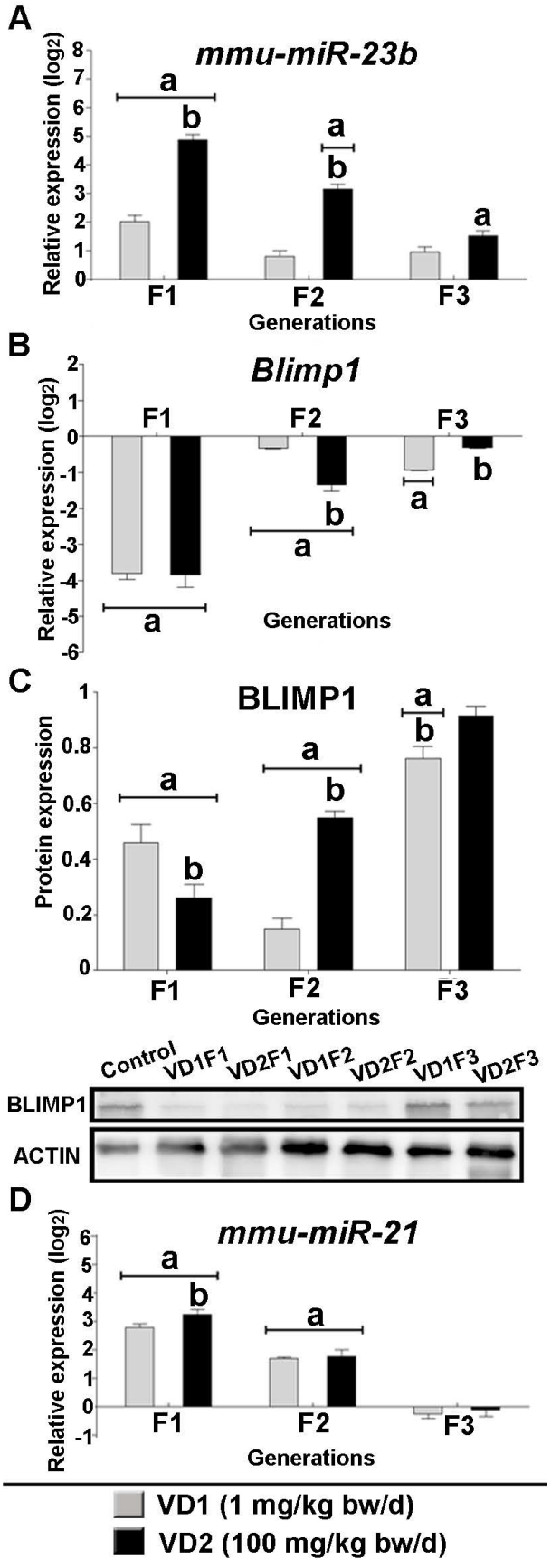
VD2 (100 mg/kg bw/d)


A
mmu-miR-23b
Relative expression (log2)
F1
F2
F3
Generations
B
Blimp1
Relative expression (log2)
Generations
C
BLIMP1
Protein expression
Control
VD1F1
VD2F1
VD1F2
VD2F2
VD1F3
VD2F3
BLIMP1
ACTIN
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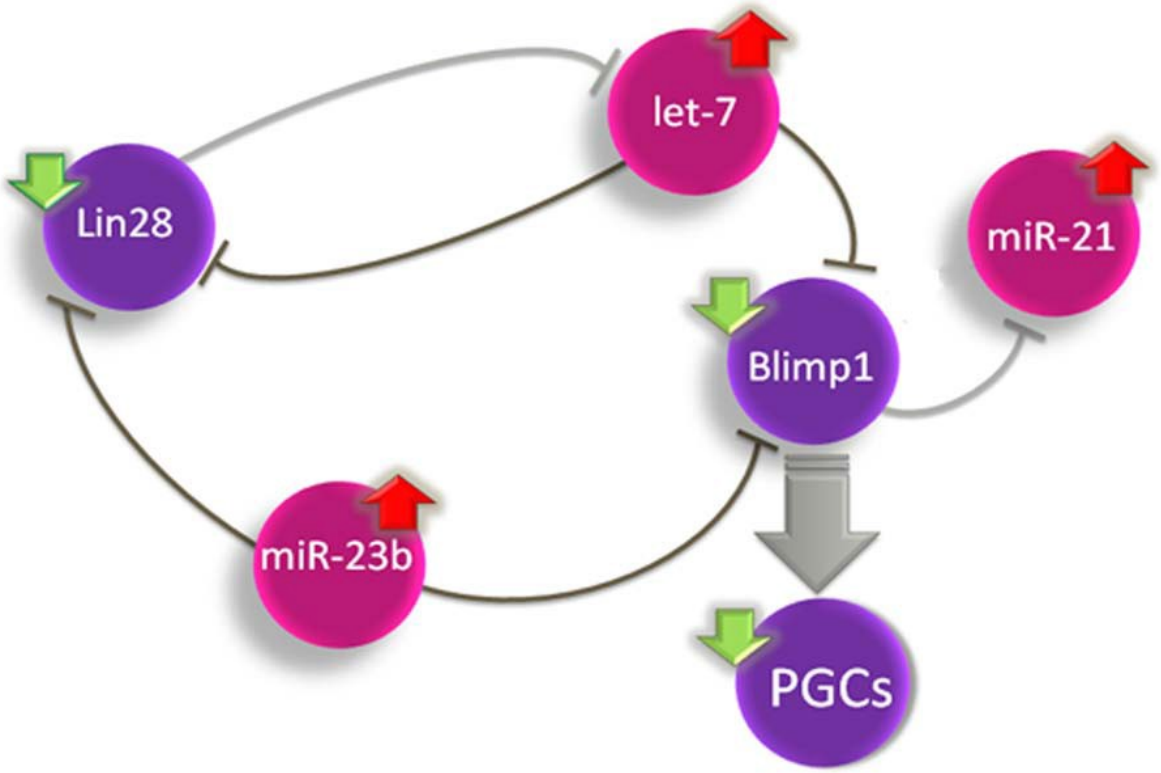
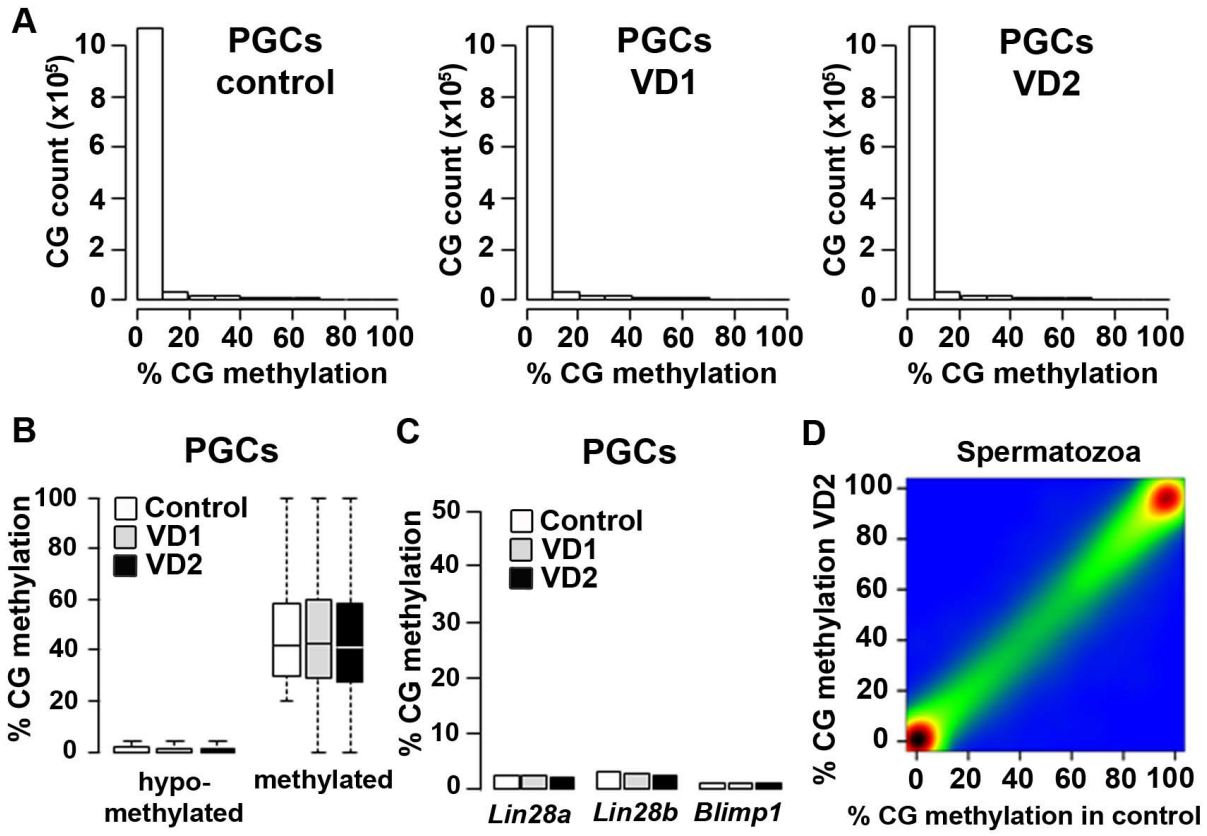
mmu-miR-21
Relative expression (log2)
VD1 (1 mg/kg bw/d)
VD2 (100 mg/kg bw/d)

Figure 5




A
PGCs control
PGCs VD1
PGCs VD2
CG count (x10^5)
% CG methylation
B
PGCs
Control
VD1
VD2
% CG methylation
hypo-methylated
methylated
C
PGCs
Control
VD1
VD2
% CG methylation
Lin28a
Lin28b
Blimp1
D
Spermatozoa
% CG methylation VD2
% CG methylation in control

S1 Material and Methods

Exposure of mice to VCZ

CD-1 mice were supplied by our own animal facility, the CIB-CSIC bioterium. The mice were bred under pathogen-free (SPF), controlled temperature (22±1 °C) and regulated humidity (50–55%) conditions with periods of light/dark of 12 h and food available at libitum. The estimated intakes of VCZ were calculated on the basis of average drinking and body weight recorded in previous experiments performed in our lab and in agreement with the data in the literature. The exposure to VCZ began the day of the vaginal plug, which is designated 0.5 dpc. The pregnant female mice treated with VCZ were designated as the F0 generation. The offspring of the F0 generation were the F1 generation. 20 adult females were used in each group to produce the F1 generation. At 13.5 dpc 10 mothers from each group were sacrificed to recover gonads from F1 fetuses for the isolation of PGCs and the histological analysis. The remaining 10 mothers were led to give birth. After eight weeks, 25 males of the F1 generation were randomly selected and bred with 50 non-exposed females (two females per male) to obtain the F2 generation. The rest of the F1 males were sacrificed to collect samples for molecular and histopathological analysis. The same process was repeated to obtain the F3 generation.

TUNEL and immunocytochemical analysis

Paraffin-embedded tissue sections were rehydrated in water, re-fixed in 4% formaldehyde, incubated in proteinase K solution (20 g/ml) for 5 min, washed two times in PBS, incubated for 10 min in an equilibration buffer and finally exposed for 1 h to the labeling buffer containing both FITC-labeled dUTP and terminal deoxynucleotidyl transferase. Control samples without the terminal deoxynucleotidyl transferase enzyme or treated by DNase were also prepared. TUNEL-labeled samples were then washed in saline–sodium citrate buffer and mounted in Vectashield with

DAPI to visualize the nuclei. TUNEL-positive cells were counted in 20 cross-sections of seminiferous tubules by fluorescent microscopy. Ten tissue slides were analysed per testis and the numbers of TUNEL-positive cells were normalized per area and averaged as technical replicates. Twelve sections from 10 different testis were analyzed in each group. In all samples, the number of TUNEL positive cells was analysed by NIS Elements picture analyser.

The embryonic testes were dissected from 13.5 dpc embryos and fixed in 4% formaldehyde in PBS. We prepared paraffin-embedded tissue section (5μM thick). PGCs were labelled by anti-SSEA1 antibody (Abcam, ab16285). In brief, the tissue sections were deparaffinized, rehydrated and incubated in PBS for 5 min. Antigen retrieval was performed by heating in citrate buffer (IHC world general protocol) and HistoReveal (Abcam) according to the manufacturer protocol (5 min incubation at RT) and protein blocking was performed by Protein Block (Abcam) during 10 min at RT. The slides were subsequently incubated with the primary antibody (anti-SSEA1, Abcam, ab16285, 10 μg/ml) at 37 °C for 1 h. After washing in PBS, slides were incubated with the secondary antibody (Goat Anti-Mouse IgM H&L, Abcam, 10 μg/ml) at 37 °C for 1h. After washing in PBS and water, the tissue slides were used for TUNEL assay according to the protocol described above with some modifications (avoiding formalin fixation and proteinase K digestion). Subsequently, slides were mounted with Vectashield (Vector) (DAPI counterstaining) and analysed by fluorescent microscopy. The numbers of TUNEL-positive and SSEA1-positive cells were counted with NIS Elements picture analyser and normalized to the sample area

PGC purification

Gonads from 13.5dpc embryos were dissected in EmbryoMax M2 Medium (Merck-Millipore), and testis were recognized from ovaries by their morphological appearance. Testis were separated from the mesonephros, disaggregated in 1000 μl of 0.25% trypsin-EDTA/20 μg/ml DNase (Sigma-Aldrich) and incubated for 20 min at

37ºC. The enzymatic reaction was stopped by adding M2 medium with 10% FCS (Gibco). The cells were centrifuged at 5000 rpm for 2 min and washed twice with 500 µl of M2 medium containing 10% FCS and 20 µg/ml DNase. The cell pellet was resuspended in 400 µl of M2 medium, mixed with 30 µl anti-SSEA-1 (CD15) MicroBeads (Miltenyi Biotech) and incubated for 45 min at 4 ºC. PGCs were isolated from stromal cells with a MS column (Miltenyi Biotech) following the manufacturer's instructions. The purity of PGCs was verified by counting cells that stained positive for alkaline phosphatase with the naphtol AS-MX/ FAST-RED (Sigma-Aldrich) following the manufacturer's instructions. The enrichment of the PGCs was 93-96%.

RT-qPCR

MicroRNAs were analysed from10ng of total RNA to prepare complementary DNA in a final volume of 15 µl, containing: 1X RT buffer, 0.25 mM of each dNTP, 50U of MultiScribeReverse Transcriptase (Applied Biosystems), 3U of RNase inhibitor and the specific stem-loop primer. The reaction was performed at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, We performed The cDNAs resulting from the reverse transcription reaction were amplified by real-time quantitative PCR with the TaqMan universal PCR master mix using the following conditions: 10 min at 95 °C followed by 40 cycles of 97 °C for 30 s and 60 °C for 1 min in the LightCycler 480 system (Roche).

mRNA expression was also performed by RT-qPCR. After heat-shock treatment at 95 °C for 5 min, total RNA (100ng) was retrotranscribed by adding 10 µl of a mix containing 2.5 µM Oligo dT17, 1X First-Strand Buffer (Invitrogen), 0.01 M dithiothreitol (DTT), 2U of RNase inhibitor (RNAsin, Promega), 0.5 mM of each dNTP and 200U of superscript II (Invitrogen). The reaction was completed to a final volume of 20 µl with RNase-free water. The cDNAs resulting from the reverse transcription reaction were amplified by real-time quantitative PCR. Reactions were performed by adding 7 µl of LightCycler 480 probes master mix (Roche) to each well

containing 3 µl of template cDNA and 0.0625 µM of each specific primer in a 10 µl reaction volume. PCR profiles were obtained using the LightCycler 480 System (Roche) using the following PCR conditions: denaturation at 95 °C for 10 min; 50 cycles of amplification of 15 s at 95 °C, 30 s at 61.4° C and 1 min at 72 °C. Synthetic oligonucleotides used for PCR were designed online with ProbeFinder version 2.20 (Roche) and purchased from Roche. Data was normalized using the 2-ΔΔCt method (1) and *Sdha* and *Mapk1* to normalize the data (2).

The following primers were used for miRNA and mRNA analyses:

Gene	Foward primer	Reverse primer
Mapk1	GGATTGAAGTTGAACAGGCTCT	GAATGGCGCTTCAGCAAT
Sdha	CAGTTCCACCCCACAGGTA	TCTCCACGACACCCTTCTGT
Tut4 (Zcchc11)	CAGCAAAGAAAGCCACCAGT	AAAAGGCATTCCATCCATCA
Tut7 (Zcchc6)	CATTAAAAAGGAATGCCCACA	TTCTTTTTGTCTTCATGTAAAAGCAC
Blimp1 (Prmd1)	TGCGGAGAGGCTCCACTA	TGGGTTGCTTTCCGTTTG
Lin28a	ACATGCAGAAGCGAAGATCC	CCTTGGCATGATGGTCTAGC
Lin28b	GAGTCCAGGATGATTCCAAGA	TGCTCTGACAGTAATGGCACTT
pri-let-7a	CCCTGGATGTTCTCTTCA	TCACCTTAGGAAAGACAGTAGATT
mmu-let-7a-1-3p	MIMAT0004620	TaqMan miRNA assay (Applied Biosystems)
mmu-mir-21	MIMAT0004628	TaqMan miRNA assay (Applied Biosystems)
mmu-mir-23b	MIMAT0012538	TaqMan miRNA assay (Applied Biosystems)

Western Blot analysis

The PGC lysates were incubated for 25 min at 4 °C, centrifuged at 13000 rpm and 4 °C during 10 min, and the supernatants were stored at -80 °C. Samples were boiled for 5 min in sample buffer, electrophoresed on SDS-polyacrylamide gels (15%) and transferred to nitrocellulose membranes. Membranes were blocked with 2% albumin (Sigma-Aldrich) in PBS plus 0.1% Tween 20 (PBS-T) at 4 °C overnight. Primary-antibody incubation was performed for 12 h at 4 °C at 1:1000 dilution of a polyclonal anti-BLIMP1 antibody (kindly gifted by Reuben Tooze, St James's University Hospital; Leeds, UK), followed by horseradish peroxidase-conjugated goat

anti-rabbit IgG or goat-antimouse (1:2500) for 1 h (R&D, HAF008). Membranes were washed in PBS-T between each step and signal-detection was carried out using the superSignal® substrate (Thermo Scientific). Images were captured with the LAS-3000 imaging-system (Fuji) and analyzed by ImageJ (http://rsbweb.nih.gov/ij).

DNA methylation

Final RRBS libraries were amplified with 17 cycles of PCR and subjected to paired-end sequencing (2x75bp) on an Illumina HiSeq2000 apparatus. Sequencing reads were cleaned with Trim Galore and aligned to the mouse mm10 genome with BSMAP, allowing 4 mismatches. We only retained reads with a unique best hit in the genome. Methylation percent values were calculated with BSMAP as the ratio of the number of Cs over the total number of Cs and Ts. For all data analysis, we filtered CpGs to have a minimum sequencing depth of 8x. RRBS was performed on pools of PGCs purified from 30 testes. For sperm, RRBS was performed in duplicates on sperm pooled from three exposed animals, as well as sperm samples pooled from unexposed animals. To find differences in DNA methylation, we averaged methylation scores in 400bp tiles and searched for tiles with at least 3 CpGs and 25% methylation difference. All data processing and representation were performed with the R software using custom developed scripts (http://www.r-project.org). For genespecific analysis of DNA methylation, we performed bisulphite conversion of genomic DNA with the Epitect kit (Qiagen). Subsequently, PCR amplifications were performed at regions of interest followed by cloning, as described (3).

References

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2. van den Bergen JA, Miles DC, Sinclair AH, & Western PS (2009) Normalizing gene expression levels in mouse fetal germ cells. Biol Reprod

81(2):362-370.

3. Borgel J, et al. (2010) Targets and dynamics of promoter DNA methylation during early mouse development. Nature Genet 42(12):1093-1100.

Supplemental Material

Figures and tables

Figure S1. Cartoon representation of the experimental model approach of VCZ exposure used and the cells, tissues and parameters assessed in each generation.

Figure S2. Expression of terminal uridylyltransferases (TUTases) in germ cell precursors of mice exposed to VCZ. The graphs show the expression of Tut4 (A) and Tut7 (B) in 13.5 dpc PGCs from VD1 and VD2 exposed embryos relative to the unexposed control (log2 of fold change of expression) along the three generations. The graph bars include standard deviation (SD), (a) indicates statistical differences of VD1 and VD2, compared to control ($p \leq 0.01$), (b) indicates statistical difference of VD1 compared to VD2 ($p \leq 0.01$). The bars include standard deviation (SD).

Figure S3. Methylation profiles in sperm from control F1 males and F1 males exposed to the high dose of Vinclozolin (VCZ). The graphs show RRBS methylation scores at single CpGs in the exón 18 of the Taok3 gene (A) and at known maternal germline DMRs (gDMRs) of imprinted loci (B). In B, the green bars depict the position of the gDMR.

Table S1. DNA methylation sequencing statistics.

Figure S1

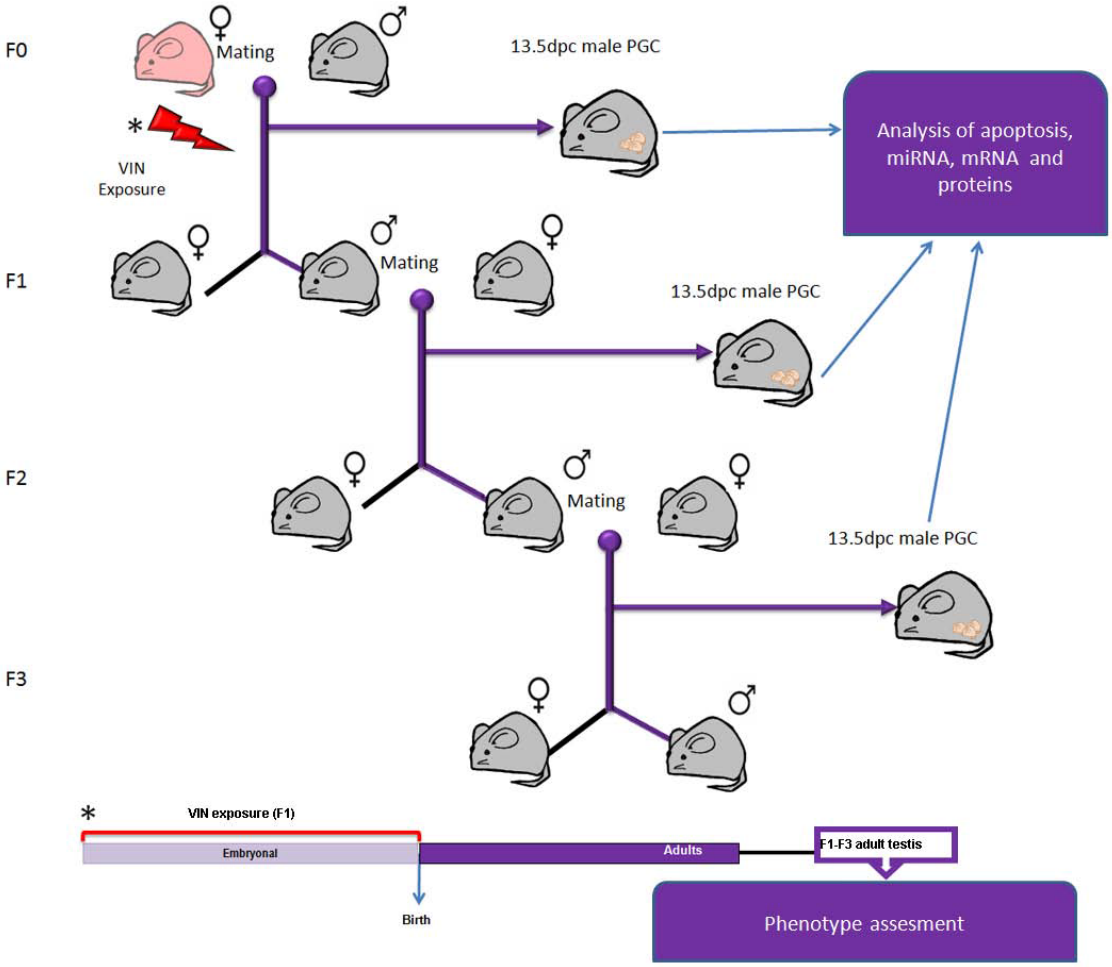
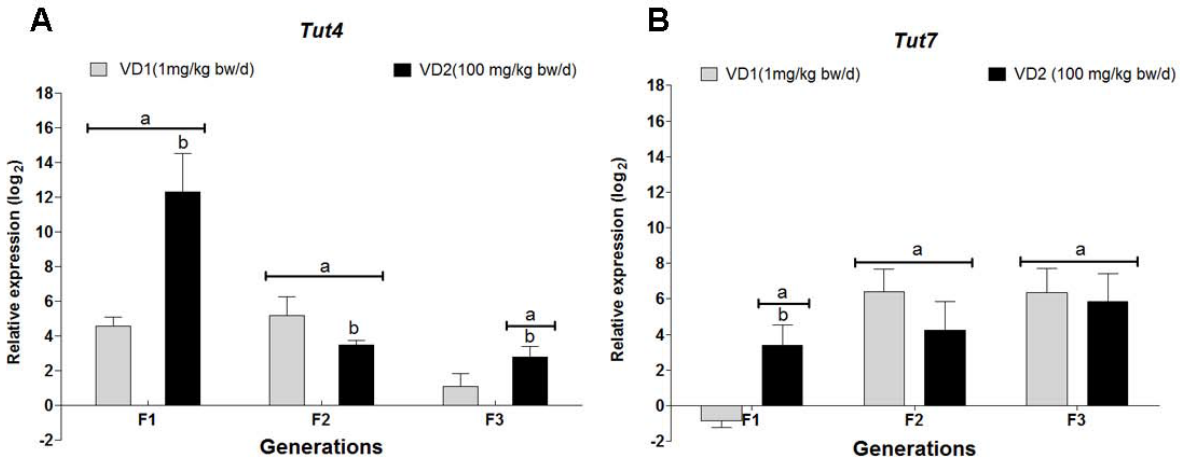

F0
Mating
13.5dpc male PGC
VIN
Exposure
Analysis of apoptosis, miRNA, mRNA and proteins
F1
Mating
13.5dpc male PGC
F2
Mating
13.5dpc male PGC
F3
VIN exposure (F1)
Embryonal
Adults
F1-F3 adult testis
Birth
Phenotype assesment

Figure S2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VD2(100 mg/kg bw/d)
Relative expression (log2)
18
16
14
12
10
8
6
4
2
0
-2
a
b
a
b
a
b
F1
F2
F3
Generations
B
Tut7
VD1(1mg/kg bw/d)
VD2 (100 mg/kg bw/d)
Relative expression (log2)
18
16
14
12
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8
6
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0
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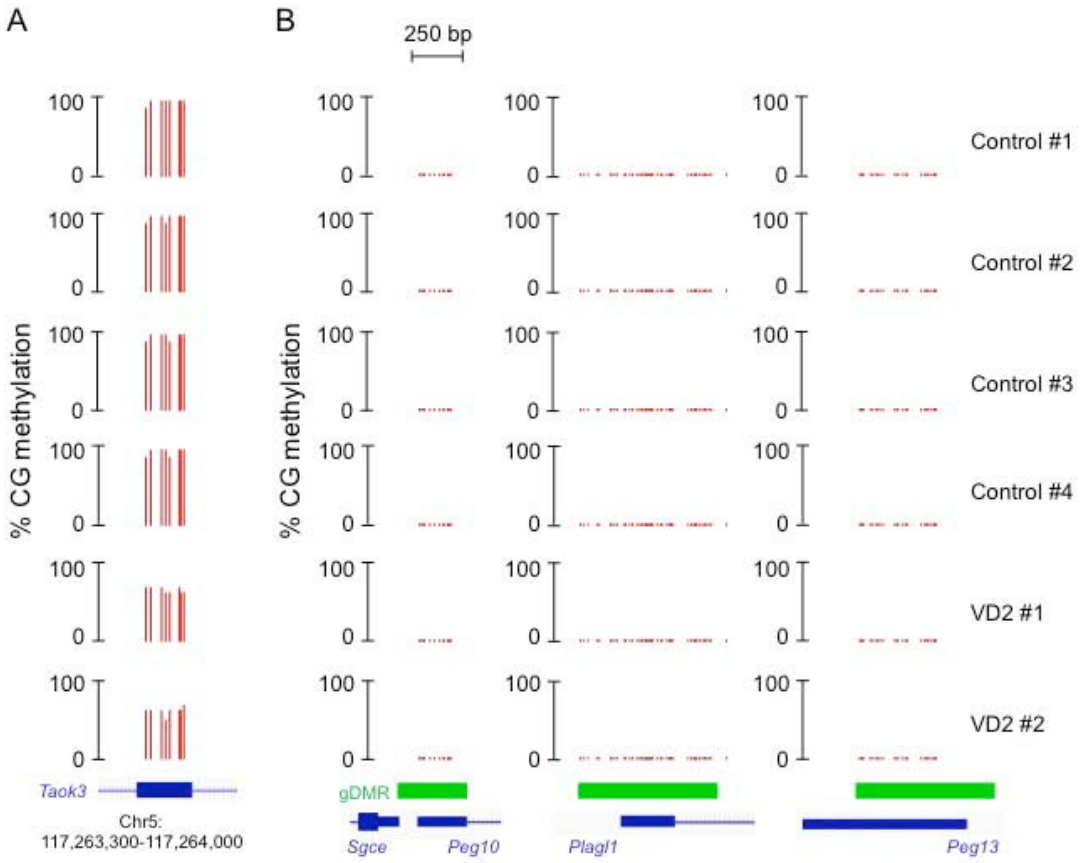
a
b
a
a
F1
F2
F3
Generations

Figure S3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% CG methylation
100
0
Control #1
Control #2
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VD2 #1
VD2 #2
Taok3
Chr5:
117,263,300-117,264,000
gDMR
Sgce
Peg10
Plagl1
Peg13

Table S1 DNA methylation sequencing statistics.

Sample	Paired-end reads	Mapping efficiency (%)	Reads with unique hits (%)	Estimated conversion rate (%)	CpGs 1x	CpGs 8x	Median sequencing depth
PGCs control#1	29,602,025	89.43	69.34	99.91	1,519,279	1,273,335	63x
PGCs control#2	26,459,394	89.31	69.88	99.88	1,501,617	1,256,928	58x
PGCs VD1	29,483,225	89.62	69.71	99.88	1,494,118	1,263,741	64x
PGCs VD2	25,952,231	90.16	70.74	99.90	1,447,293	1,220,629	55x
Sperm control#1	41,614,839	92.51	71.29	99.67	1,506,335	1,285,697	80x
Sperm control#2	27,370,903	92.39	71.59	99.78	1,474,147	1,232,252	58x
Sperm control#3	23,916,584	92.71	70.31	99.79	1,477,737	1,241,280	49x
Sperm control#4	24,186,087	92.14	72.44	99.80	1,532,820	1,234,934	46x
Sperm VD2#1	26,074,125	92.11	72.79	99.77	1,523,119	1,295,320	56x
Sperm VD2#2	29,206,596	89.26	70.81	99.76	1,545,102	1,260,722	53x

List of publications

Publications related to this thesis:

1. **Ded, L, P Dostalova, A Dorosh, K Dvorakova-Hortova, and J Peknicova** 2010 Effect of estrogens on boar sperm capacitation in vitro. *Reproductive Biology and Endocrinology* **8**.
   IF (2012) = 2.144, IF (2010) = 1.695
2. **Sebkova, N, M Cerna, L Ded, J Peknicova, and K Dvorakova-Hortova** 2012 The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens. *Reproduction* **143** 297-307.
   IF (2012) = 3.555
3. **Ded, L, N Sebkova, M Cerna, F Elzeinova, P Dostalova, J Peknicova, and K Dvorakova-Hortova** 2013 In vivo exposure to 17 beta-estradiol triggers premature sperm capacitation in cauda epididymis. *Reproduction* **145** 255-263.
   IF (2012) = 3.555
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